1991

T lymphocyte reactivity and antibody response to a synthetic peptide (glutamic acid-alanine-tyrosine) as a marker for disease resistance in chickens

Edward Merrill Steadham
Iowa State University

Order Number 9212194

T lymphocyte reactivity and antibody response to a synthetic peptide (glutamic acid-alanine-tyrosine) as a marker for disease resistance in chickens

Steadham, Edward Merrill, Ph.D.

Iowa State University, 1991

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

T lymphocyte reactivity and antibody response to a synthetic peptide (glutamic acid-alanine-tyrosine) as a marker for disease resistance in chickens

by

Edward Merrill Steadham

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major: Immunobiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Program

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1991

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

APC - antigen-presenting cell

CD - cluster of differentiation

Con-A - concanavalin A

DMEM - Dulbecco's modified Eagle's medium

Ea-B - erythrocyte alloantigen, chicken blood group locus

GAT - poly(glutamic acid^{60}-alanine^{30}-tyrosine^{10})

GVH - graft versus host

IgG - immunoglobulin G

IL-2 - interleukin 2

Ir-GAT - gene or genes responsible for humoral immune response to GAT

Kd - kilodalton

MD - Marek's disease

MDV - Marek's disease virus

MG - *Mycoplasma gallisepticum*

MHC - major histocompatibility complex

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NOR - nucleolar organizer region

PBS - phosphate buffered saline

PHA - phytohemagglutinin

PM - *Pasteurella multocida*

RBC - red blood cell

RFLP - restriction fragment length polymorphism

RSV - Rous sarcoma virus

INTRODUCTION

Among genetic markers linked to disease resistance in chickens, the major histocompatibility complex (MHC) is the most widely studied. Chickens with particular MHC alleles have been found to be more resistant to Marek's disease (Hanson *et al.* 1967, Steadham *et al.* 1987), Rous sarcoma virus-induced tumors (Collins *et al.* 1977, Schierman *et al.* 1977) and fowl cholera (Lamont *et al.* 1987). Adult mortality levels have also been linked to the MHC in chickens (Nordskog *et al.* 1977). Humoral immune response, as measured by antibody titer produced in response to challenge with the amino acid polymer GAT [Poly (Glu^{60} Ala^{30} Tyr^{10})], has also been associated with the chicken MHC (Benedict *et al.* 1975). These observations are not surprising, because the gene products of the MHC contribute to recognition and interaction among cells of the immune system (Clark 1983, Klein 1986). Genetic selection for improved general disease resistance through selection for immune response characteristics or markers is an attractive possibility and has the advantage of not risking valuable genetic stock by direct disease challenge (Gavora and Spencer 1983).

Immune response (*Ir*) genes were first described in outbred lines of guinea pigs (Levine *et al.* 1963). The ability to respond (as measured by antibody production) when challenged with dinitrophenyl-poly-L-lysine was found to be under control of a single autosomal dominant gene. The availability of inbred mouse strains enabled McDevitt and Chintz (1969) to link response to the branched amino acid polymer (T,G)-A-L to the mouse

MHC. Recombinants within the mouse MHC allowed for the mapping of the *Ir-*(T,G)-A-L gene within the *I* region of the MHC (McDevitt *et al.* 1972).

Benedict *et al.* (1975) described the close association between immune response to GAT (*Ir-GAT*) and the chicken *B* complex (MHC). Pevzner *et al.* (1978) reported on the recombination between the gene(s) for *Ir-GAT* and the serologically determined MHC genes (*Ea-B* or *B-F/B-G*) in the Iowa State University S1 line of chickens. Based on structural and functional homology, the gene products of the *B-L* region of the chicken MHC are the equivalent of the mouse MHC *I* region (Hala *et al.* 1981). The original hypothesis was that the dichotomy between *Ea-B* and *Ir-GAT* could be due to a recombinational event between the *B-F/B-G* subregions and the *B-L* subregion of the chicken MHC.

Attempts to detect evidence of chromosomal recombination in the S1 line expressed as a difference in cell surface proteins have proven unsuccessful. Restriction fragment length polymorphisms (RFLP) generated by probing S1 line DNA with a *B-L* gene probe have been associated with *Ea-B* differences (Pitcovski *et al.* 1989). No RFLP were associated with differences in *Ir-GAT*. It appears that humoral immune response to GAT in the S1 line could be determined by mechanisms of cell interaction other than those mediated through class II molecules. Possibly the contributions of the T cells to immune response (subject to MHC restriction) could be a determining factor. The research presented in this dissertation intends to clarify the nature of the antibody response to GAT in the S1 line of chickens by examining the response of antigen-primed lymphocytes when exposed to GAT *in vitro*.

LITERATURE REVIEW

A large body of work has accumulated describing the details of the chicken major histocompatibility complex (MHC). The earliest papers describe the biochemical aspects of the gene products. More recent work describes the genomic organization of the complex. The details emerging from these studies will be discussed in the following review. In addition, the Iowa State S1 line of chickens has been widely used in immunogenetic studies, many of which describe the association of particular MHC alleles with resistance or susceptibility to diseases of economic impact in poultry. The relationships of humoral immune response to glutamic acid-alanine-tyrosine (Ir-GAT) and various poultry diseases and immune response characteristics have also been reported in studies using the S1 line of chickens. These studies indicate the importance in understanding the underlying mechanisms that could account for the differences in response to GAT, as well as the differences in disease susceptibility. While the mechanisms involved in immune response are well-studied in mammals, progress in understanding the mechanisms in non-mammalian species is at a much lower level.

The Chicken Major Histocompatibility Complex (*B* Complex)

Based on functional and structural homologies to mammalian systems, the B complex is the chicken major histocompatibility complex (MHC) (Pazderka *et al.* 1975a). The *B* complex was first described as a gene (*Ea-B*) controlling an alloantigen expressed on the surface of chicken

erythrocytes (Briles *et al.* 1950). In 1961, Schierman and Nordskog reported on the linkage of *Ea-B* and the gene(s) controlling skin graft acceptance or rejection. Later, Miggiano *et al.* (1974) demonstrated that the mixed lymphocyte reaction (the proliferative response of cells when mixed in culture with non-self cells) in chickens is controlled by the genes of the *B* complex. The close association of *Ea-B* and histocompatibility led to the chicken MHC being named the *B* complex.

The major elements of the *B* complex can be explained by the three locus model proposed by Pink *et al.* (1977). Based on tissue distribution and functional and biochemical analyses, there are three types of antigens or antigenic complexes that are genetically coded within the chicken MHC. These antigens are expressed on the surface of cells as transmembranous proteins. The three types of gene products are the B-F, B-L, and B-G antigens.

The *B-F* (Class I) gene products have the widest tissue distribution throughout the chicken and are found on most somatic cells, peripheral blood lymphocytes (PBL), and red blood cells and their precursors (Pink *et al.* 1977, Hála *et al.* 1981). The *B-F* gene product is a 40-45 Kd glycoprotein that is non-covalently associated on the cell surface with β_2 microglobulin, which is coded for outside of the *B* complex (Pink *et al.* 1985). The B-F is the apparent homologue of the mouse MHC Class I (K or D) antigen and as such is a highly polymorphic classical transplantation antigen.

The *B-L* (Class II) region gene products have a narrower cellular distribution pattern. The B-L antigens are found on B cells and cells of

monocyte/macrophage lineage (Hála *et al.* 1981). As expressed on the cell surface, the B-L antigen is a transmembranous non-covalent heterodimer composed of a relatively invariant α chain (32-34 Kd) and a polymorphic β chain with an apparent molecular size of 27-29 Kd (Ewert *et al.* 1984, Guillemot *et al.* 1986). It is widely accepted that the *B-L* region of chickens is the homologue of the mouse MHC *I* region or the human MHC *D* region (Hála *et al.* 1981).

The *B-G* (Class IV) region codes for the polymorphic alloantigens found primarily on erythrocytes and their precursors. There are no known mammalian homologues for the *B-G* region gene products and there are no known immunological functions associated with B-G antigens. As expressed on the surface of erythrocytes, the B-G antigen is a disulfide-linked homodimer (40-48 Kd/chain) and is not glycosylated (Salomonsen *et al.* 1987, Morgan *et al.* 1990). The variation in apparent molecular size may be due to the length of the cytoplasmic portion of the molecule (Guillemot *et al.* 1989). The presence of B-F and B-G antigens on erythrocytes forms the basis of MHC typing in chickens by hemagglutination with alloantisera.

Genomic organization of the *B* complex

The molecular map of the chicken MHC is far from complete, but recent reviews have pointed out some homologies and some differences compared to the mammalian MHC (Guillemot *et al.* 1989, Kroemer *et al.* 1990). Sequence homology between a human MHC Class II β gene and chicken *B-L* β provided an entry point into the chicken MHC. Subsequently, specific chicken *B-F* α, *B-L* β, and *B-G* gene probes were developed and

characterized by chromosome walking. These probes were in turn used to identify and map MHC genes on four non-overlapping cosmids constructed from chicken DNA of the B^{21} haplotype. The cosmids covered 320 Kb of the chicken MHC and nucleolar organizer region (NOR). The NOR contains the DNA for two ribosomal RNA genes and has been linked to the *B* complex (Bloom and Bacon 1985) and marks the telomeric end of the chicken MHC (Guillemot *et al.* 1988). The distances between Class I (*B-F*) and Class II (*B-L*) genes are extremely small when compared to mammalian MHC. The four cosmids also contained more than one boundary between Class I and Class II genes, indicating that they are interspersed along the length of the complex. The compactness of the coding regions and their arrangement may account for the failure to detect recombination between Class I and Class II genes often reported in the literature (Skjødt *et al.* 1985, Hála *et al.* 1988). Unlike the mammalian MHC, there do not appear to be Class III genes (coding for some complement serum proteins) within the chicken MHC (Guillemot *et al.* 1988).

There are also non-Class I and non-Class II genes scattered among the *B-F* and *B-L* genes, at least some of which are transcribed to mRNA. The function of these genes is not known. Kroemer *et al.* (1990) have speculated that they may be equivalent to mouse *Qa/Tl* genes (Class I). One of the transcribed genes appears to code for a subunit of the GTP-binding protein (G protein) which is not in the mammalian MHC (Guillemot *et al.* 1989a). The G proteins function in transmembrane signalling in immune cells (as well as other cell types) and thus could have immunological significance (Neer and Clapham 1988).

The ISU S1 Line of Chickens

The S1 line of Leghorn chickens originated at Iowa State University from a cross of two inbred commercial white Leghorn lines in 1963 (Nordskog *et al.* 1973). Selection at that time was based solely on the B blood group (which marked the MHC type); all combinations of homozygotes and heterozygotes were produced each generation. There was no selection based on performance or viability traits. Full and half-sib matings were avoided. The original alleles segregating in the S1 line were B^1, B^2, B^{19}, and B^{21}.

Since the foundation of the S1 line, the B^{21} MHC haplotype has been dropped from the matings and the chickens have been maintained as sublines since 1978 (Pevzner *et al.* 1978). The sublines are based on their MHC type (B^1 or B^{19}) and their humoral immune response (high or low) when challenged with GAT. The B^2 haplotype has been maintained as a control within the S1 line. Matings within the five sublines (Table 1) were random until 1983. Since that time the sublines have been maintained with avoidance of full or half-sib matings.

Immunogenetic studies in the ISU S1 line of chickens

There have been many immune response and disease resistance studies conducted using the S1 line. In 1972 and 1973, S1 line hens (MHC types B^1B^1, B^1B^2, or B^1B^{19}) were inoculated with *Salmonella pullorum* bacterin and their immune response was measured by agglutination assay (Pevzner *et al.* 1975). The B^1 homozygotes were significantly lower antibody producers than the B^1 heterozygotes. A divergent selection experiment within the B^1

Table 1. Blood types and GAT response designations for S1 line MHC haplotypes used in this dissertation

Blood Type	GAT Response	Haplotype Designation	Alternative Designation
19	High	19H	B^{19H}
19	Low	19L	B^{19L}
1	High	1H	B^{1H}
1	Low	1L	B^{1L}
2	Intermediate	2	B^{2}

haplotype for response to *S. pullorum* indicated that a fourfold difference in antibody titer could be reached after three generations of selection (Pevzner *et al.* 1977). The genetic basis was thought to be polygenic with perhaps as few as one gene linked to the MHC. High adult mortality and lower egg production had also been associated with the B^1 homozygotes (Nordskog *et al.* 1973) and was thought to demonstrate the pleiotropic nature of the MHC or linkage to a viability or fitness genes.

In 1978, Pevzner *et al.* reported evidence of recombination between the serologically detectable MHC gene products (B-G and B-F) and the gene or genes responsible for immune response to GAT (*Ir-GAT*). When immunized with GAT, the secondary immune response as measured by radioimmunoassay (RIA) was clearly associated with the *B* complex. The B^1 homozygotes were low or non-responders ($Ir\text{-}GAT^L$) and the B^{19} homozygotes were high responders ($Ir\text{-}GAT^H$) (5.2 and 53.1% GAT bound respectively). The appropriate backcrosses indicated linkage between immune response to GAT

and the *B* complex and that high response was a dominant trait. The percentage of GAT bound was different in the backcross chickens. For example, the B^1 homozygotes derived from $B^1/B^1XB^1/B^{19}$ matings averaged 16.2% GAT bound. There were exceptional individuals whose response was like that of the other MHC type (*i.e.* B^1B^1 *Ir-GAT*^{H}). Progeny testing of these chickens met expectations of Mendelian segregation and lent support to the hypothesis of recombination between *Ea-B* (the region coding for the serologically detectable MHC proteins) and *Ir-GAT*, the locus responsible for response to GAT.

It should be noted that the S1 line was maintained at this time as random-bred MHC heterozygotes and homozygotes that would allow recombination. There was also the possibility that *Ir-GAT*^{2} could have been involved in a recombination, but the immune response to GAT in the B^2 haplotype chickens was not as clearly defined as the B^1 or the B^{19} *Ir-GAT*.

The immune responses to other antigens were also examined in the S1 line. The B^1 homozygotes were also low responders (as measured by antibody production) to (T,G)-A-L (a branched amino acid polymer of tyrosine, glutamic acid, alanine, and lysine) and human serum albumin (HSA) and GT (a linear amino acid polymer of glutamic acid and tyrosine). Chickens that were of the B^{19} haplotype were high responders to (T,G)-A-L and HSA and low or non-responders to GT (Pevzner *et al.* 1979).

From 1978 to the present, the S1 line has been maintained as sublines through intra-haplotype mating by forced segregation for the serologically detectable MHC antigens and *Ir-GAT* type. Immunogenetic studies using the S1 line since the creation of the sublines have

exploited *Ir-GAT* as an additional genetic marker and various disease response traits have been associated with the locus. In 1979, Gebriel *et al.* determined that chickens within the B^1 haplotype were more resistant to Rous sarcoma virus (RSV) induced tumor development if they were also high responders to GAT. A subsequent study (Gebriel and Nordskog 1983) using S1 chickens of the B^{19} haplotype that had been characterized for GAT response provided additional evidence that the *Rs* locus controlling tumor regression was linked to *Ir-GAT*. Again, GAT low responder chickens were more susceptible to tumor progression.

Previous investigators had also shown that the MHC was linked to the gene or genes controlling outcome of RSV-induced tumors (Collins *et al.* 1977, Schierman *et al.* 1977). Gebriel's study suggested that the response to the RSV-induced tumors was at least partially controlled by a gene linked to the *Ir* region of the MHC, assuming that the *Ir-GAT* gene was found in that region.

The *I* region genes of the mouse MHC were identified as immune response genes and Ia antigens (their gene product) were found while attempting to identify immune response gene products serologically through reciprocal immunizations between high and low responders to antigen (Shreffler and David 1975). Caution should be exercised when comparing mouse immune response mechanisms to those of chicken immune response. The limited antigen diversity of linear amino acid polymers insures that most inbred mouse lines either respond or do not respond (Benacerraf and Dorf 1977). Chickens are not non-responders to GAT but are low responders (Benedict *et al.* 1975).

Chickens representing the four sublines, as well as the within line B^2/B^2 control, were challenged by inoculation with the JM strain of Marek's disease virus (Pevzner *et al.* 1981). Low responders to GAT were more susceptible to Marek's disease. Both B^1/B^1 and B^{19}/B^{19} had an incidence of the disease slightly above fifty percent. The *Ir-GAT*^{H} chickens within each blood type had a significantly lower incidence of the disease. Chickens of the B^1/B^1 *Ir-GAT*^{H} group had no mortality from Marek's disease. Mortality of the B^{19}/B^{19} *Ir-GAT*^{H} chickens was significantly higher than zero but still lower than the *Ir-GAT*^{L} chickens. In other lines of chickens the B^{19} haplotype had previously been identified as being particularly susceptible to the effects of Marek's disease (Pazderka *et al.* 1975).

A similar Marek's disease challenge was conducted on subsequent generations of S1 line chickens (for complete text see Appendix). Two sequential generations and a later F_2 population were challenged with the JM strain of MDV and mortality due to the disease and incidence of Marek's induced tumors was recorded (Steadham *et al.* 1987). Results confirmed Pevzner's observation that the *Ir-GAT*^{H} chickens had lower incidence of disease and that the B^{19}/B^{19} chickens were more susceptible than the B^1/B^1 regardless of their GAT response phenotype.

The F_2 chickens were generated by first crossing B^{19H} and B^{1L}, as well as B^{19L} and B^{1H}, to produce an F_1 population. The sublines were then rederived by *inter se* matings of the F_1 to produce the F_2 population. Chickens of the F_2 generation were then challenged with MDV. Five genetic types were generated: B^1/B^1 *Ir-GAT*^{H}, B^1/B^1 *Ir-GAT*^{L}, B^{19}/B^{19} *Ir-GAT*^{H},

B^{19}/B^{19} -*Ir-GAT*^{L}, and B^{1}/B^{19} *Ir-GAT*^{H}. The B^{1H}/B^{1H} group was significantly different in resistance to Marek's disease. The data from this trial confirmed the increased MD resistance of chickens possessing the B^{1}/B^{1} blood type when associated with the genes for high immune response to GAT.

These results suggested the possible role for complementary gene loci linked to the MHC and *Ir-GAT* gene. Reassortment of complementary gene loci would have likely occurred in generating the F_2 population. This also concurs with the assumption that immune response to GAT is in part under polygenic control with at least one gene within or closely linked to the MHC (Benedict *et al.* 1975).

There has been one disease challenge study in the S1 line using a bacterial pathogen (Lamont *et al.* 1987). This was the first instance in which the MHC was linked to resistance to a bacterial disease. *Pasteurella multocida*, the bacterium that causes fowl cholera, was inoculated intramuscularly into three-week-old chicks. The chicks used were assayed for B-G alloantigen type (B^{1} or B^{19}) and were characterized for *Ir-GAT* as well as response to RSV-induced tumor (regressor phenotype or progressor phenotype). Genetic control of resistance to high doses of *P. multocida* was associated with RSV type and *Ir-GAT* type. Both RSV regressors and *Ir-GAT*^{L} type chickens better survived challenge. However, the strain and dose of bacterium was such that mortality levels reached 87% in 24 hours. Two additional replicates of the experiment were conducted with a lower dose of inoculum. The Ea-B type was associated with resistance in these trials. The B^{1} type survived challenge better than the B^{19}. Neither GAT response nor RSV type had a significant effect

on survivability. These results were confirmed when F_1 heterozygotes and F_2 homozygotes and heterozygotes were challenged.

Assays measuring immunological response traits have also been conducted with S1 line chickens. A study published in 1981 (Rees and Nordskog) reported on the maturation of basal serum immunoglobulin G (IgG) levels through 21 weeks of age. Chickens that were of the high responder type reached greater concentrations of IgG than *Ir-GAT*^{L} chickens. Analysis of variance on results across other lines and haplotypes indicated that line effects were significant, but a genetic component was still controlled by the MHC.

Experiments were also undertaken to identify which MHC subregions in the S1 line are involved in cell-mediated alloantigen reactions, specifically the graft versus host (GVH) reaction (Lee and Nordskog 1981). Splenomegaly (spleen enlargement) is induced in immuno-incompetent chick embryos when immunologically competent lymphocytes of different MHC type are injected intravenously (Jaffe and McDermid 1962). Strong GVH reactions in mice are due to differences in the *I* region (Class II) and weaker, but still significant, reactions are induced when there are differences in the *K* or *D* (Class I) region of the MHC (Klein and Park 1973). In the S1 line, splenomegaly was greatest when there were differences in the B blood group antigens (presumably, *B-G/B-F*) and differences were smaller, albeit significant, when there were *Ir-GAT* differences between host and donor.

A series of immunocompetence measurements were taken on S1 line chickens by Cheng and Lamont (1988). Humoral immune response to

immunization with *P. multocida* (PM) and *Mycoplasma gallisepticum* (MG) bacterins was measured by enzyme-linked-immunosorbant-assay (ELISA). A carbon clearance assay was used to measure *in vivo* phagocytic ability. Phytohemagglutinin (PHA) was injected intradermally in the wing web to evaluate T cell mediated response (recruitment and proliferation) *in vivo* as measured by swelling. The three aspects of the immune response (humoral, cellular, and phagocytic ability) were thus determined for the same S1 line chickens. *Ir-GAT*^{H} chickens were significantly higher in their response to both bacterins. Response to MG vaccine was also higher in the B^{1} chickens compared to the B^{19} chickens. Phagocytic ability showed significant differences within sex. Roosters differed by blood type (Ea-B) and hens differed by Ir-GAT type. The response to PHA was also significantly different by sex. The blood type and the RSV type (regressor or progressor, based on response to RSV-induced tumor development) were significant sources of variation in the response of tested roosters. Roosters of the B^{1} blood type responded higher and so did chickens of the RSV progressor phenotype. Neither of these effects were significant in the hens tested by PHA injection. The complexity of the underlying mechanisms of the immune system makes explanation difficult, but sex differences for other immune responses have been reported. For example, wattle reaction to injection with *Staphylococcus aureus* antigen differs by sex (Cotter *et al.* 1987).

An additional measurement of immunocompetence was assayed in the S1 line by Knudtson *et al.* (1990). Interleukin 2 (IL-2, also known as T Cell Growth Factor) is produced by T cells in response to exposure to antigen,

mitogen, or IL-2 itself. In general, IL-2 enhances activity of immune cells through induction of proliferation of effector cells (Farrar *et al.* 1982). Concanavalin A (Con-A, a mitogen) was used *in vitro* to induce peripheral blood lymphocytes to secrete IL-2 into their culture medium. The resulting medium was then assayed for IL-2 activity *in vitro* with responder cells. In this case, IL-2 production was associated with Ea-B type of the producer cell and Ir-GAT type had no significant effect. Con-A-induced IL-2 production was higher for cells from B^1 haplotype chickens.

Many of the studies using S1 line chickens have demonstrated the importance of Ir-GAT as an additional genetic marker for disease resistance ability or immunological response traits. Studies using commercial lines of chickens (unrelated to the S1 line) have shown that selection for Ir-GAT does not effect performance traits (Pevzner *et al.* 1989a), but the Ir-GAT^H chickens are more resistant to RSV-induced tumors, Marek's disease, and *Staphylococcus aureus* infections (Pevzner *et al.* 1989). The original paper (Pevzner *et al.* 1978) reported that Ir-GAT was distinct from the serologically detectable MHC subregions (*B-F/B-G*) in the S1 line. By analogy to other species, *Ir-GAT* would be contained within the chicken MHC. However, it had never been definitively shown that *Ir-GAT* is within the chicken MHC. Despite intensive investigations, there has never been any serological evidence of recombination resulting in a different Class II protein being expressed on the surface of Class II-positive cells that would account for the GAT response differences seen in the S1 line. Reciprocal immunizations with class II positive cells

between high and low responder chickens have never resulted in an alloantiserum that discriminates between Ir-GAT haplotypes.

Pitkovski *et al.* (1989) attempted to detect evidence of chromosomal recombination between Ea-B and Ir-GAT in the S1 line through the analysis of restriction fragment length polymorphisms (RFLP). DNA from the S1 sublines was digested with restriction endonucleases and electrophoresed on agarose gels. After transfer to nitrocellulose, the resulting blots were hybridized with a ^{32}P-labelled Class II genomic probe (234 base pairs of the β_2 exon of the chicken class II gene, Bourlet *et al.* 1988).

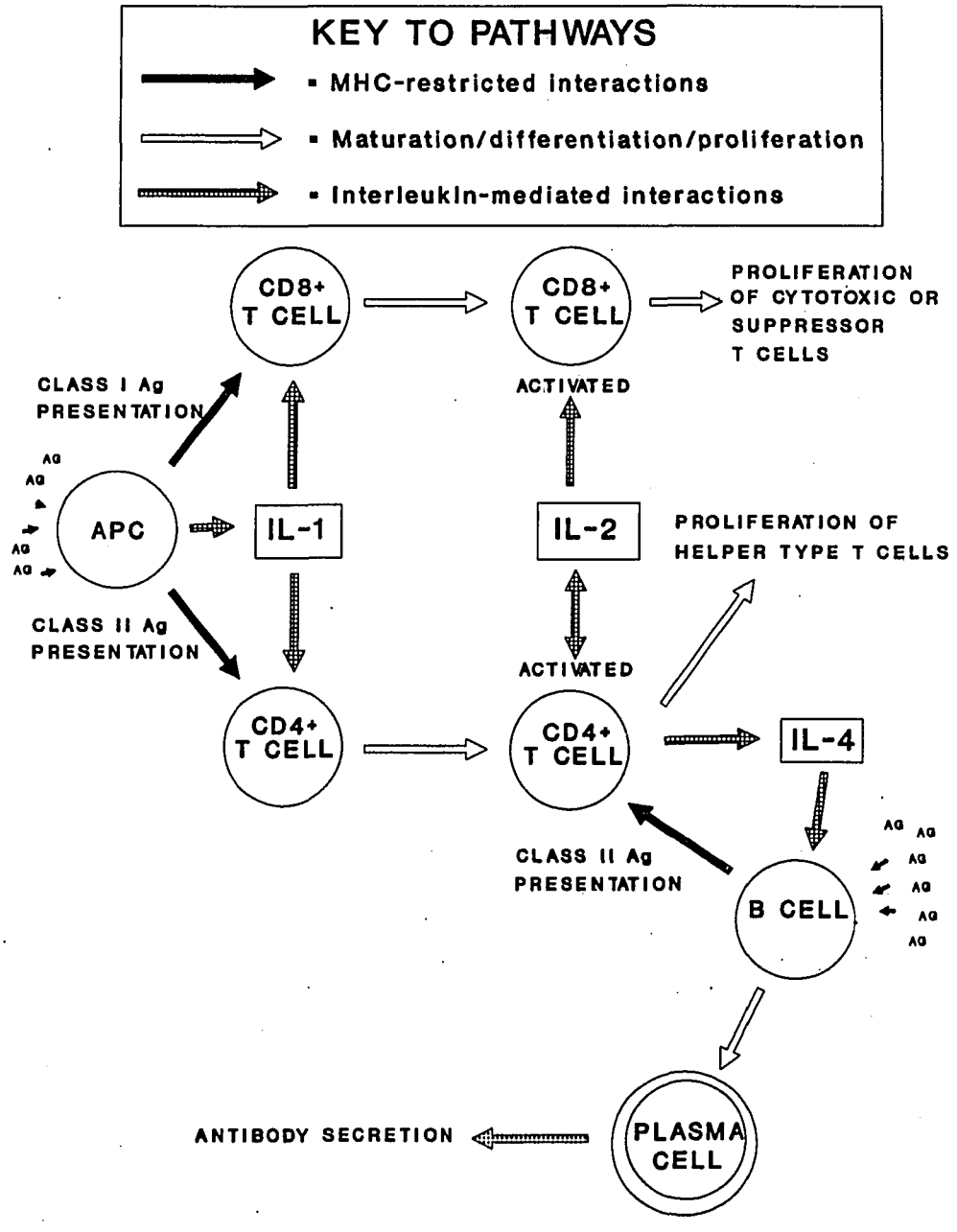
RFLP generated by 15 different enzymes failed to produce any patterns that could be associated with Ir-GAT. RFLP, generated by three enzymes of ten tested, were associated with Ea-B. Response to RSV challenge was not associated with RFLP. Three reasons for the lack of Ir-GAT association with RFLP were possible. The choice of enzymes may have excluded the polymorphic locus or loci, *Ir-GAT* is outside of the MHC, or control of immune response to GAT is mediated through the class II *B-L* α chain or the class I *B-F* molecule. Lack of association of *Ir-GAT* with class I genes was seen when S1 line DNA was examined with a class I (*B-F*) gene probe (Chen and Lamont 1991).

Antigen-Specific Immune Response Leading to Antibody Production

Figure 1 presents a schematic flow chart of the series of reactions that occur prior to antibody production in response to an antigen (based on Marrack and Kappler 1986). Antigen is phagocytosed by the antigen-presenting cell (APC, usually a macrophage), processed or digested into

Figure 1. Accessory cell and interleukin-mediated interactions in an immune response leading to antibody production

Ag, antigen; APC, antigen presenting cell; IL-1, interleukin 1; IL-2, interleukin 2 (T Cell Growth Factor); IL-4, interleukin 4 (B cell Stimulating Factor-1); MHC, major histocompatibility complex; CD8, cell surface differentiation molecule on cytotoxic/suppressor T cells; CD4, cell surface differentiation molecule on helper T cells.


KEY TO PATHWAYS
▪ MHC-restricted interactions
▪ Maturation/differentiation/proliferation
▪ Interleukin-mediated interactions
CD8+ T CELL
CD8+ T CELL
PROLIFERATION OF CYTOTOXIC OR SUPPRESSOR T CELLS
ACTIVATED
CLASS I Ag PRESENTATION
Ag
Ag
Ag
Ag
APC
IL-1
IL-2
PROLIFERATION OF HELPER TYPE T CELLS
CLASS II Ag PRESENTATION
ACTIVATED
CD4+ T CELL
CD4+ T CELL
IL-4
CLASS II Ag PRESENTATION
B CELL
Ag
Ag
Ag
Ag
Ag
Ag
ANTIBODY SECRETION
PLASMA CELL

smaller pieces, and expressed on the cell surface in association with autologous class I or class II MHC molecules. MHC restriction is the requirement for identity between Class I or Class II alloantigens on immune cells for cooperation to occur. MHC restriction of chicken immune cells has been demonstrated for interaction between T cells and B cells, as well as the reactions between T cells and antigen presenting cells (APC) (Vainio *et al.* 1983, Vainio *et al.* 1988).

Antigen processing and presentation

T lymphocytes react to most protein antigens, not in native form, but after antigen has been processed within phagocytic cells and re-expressed on the surface of the cell in association with Class I or Class II MHC proteins (Vainio and Lassila 1989, MacDonald and Nabholz 1986).

Antigen processing and presentation is in large the function of the macrophages (reviewed by Unanue 1984) due to their ability to express class I and class II MHC molecules, process antigen (ingest and re-express on the cell surface), and to produce interleukin-1 (a monokine that augments T cell proliferative response to antigen or mitogen). There is some debate over the identity of the principal APC *in vivo* and whether B cells or monocyte/macrophages are more prominent (Ashwell 1988, Kurt-Jones *et al.* 1988). Work conducted with chickens has indicated that B cells are unable to activate resting T cells *in vivo* until antigen presenting non-B cells provide a triggering signal (Lassila *et al.* 1988, DeFranco 1988). The APC presents the processed antigen to the two major subsets of T lymphocytes, the $CD4^+$ cells and the $CD8^+$ cells (for a review of T cell

activation, see MacDonald and Nabholz 1986).

T cell subsets

There are subsets of T lymphocytes based on their functional capabilities and on differentiation proteins expressed on their membrane surface. Mature peripheral T cells are divisible into two distinct populations. One set of cells expresses the CD8 receptor and the other expresses the CD4 receptor and their existence on mature cells is mutually exclusive (Chan *et al.* 1988, Littman 1987). CD refers to "cluster of differentiation". CD4 and CD8 are transiently expressed together on thymocytes as part of the maturation process of T cells (Littman 1987, Bierer *et al.* 1989).

CD4 is a phenotypic marker on helper T cells, as well as T lymphocytes involved in delayed-type hypersensitivity. Monoclonal antibodies have been developed that identify CD4 on chicken lymphocytes (Chan *et al.* 1988, Lillehoj *et al.* 1988). Lymphocytes treated with anti-CD4 and complement and subsequently cultured lose their ability produce IL-2 upon exposure to pokeweed mitogen *in vitro*. This confirms that the antibodies identify T helper cells, because the function of T cells of the helper type are to produce IL-2 upon exposure to antigen or mitogen to augment immune response.

The second population of T lymphocytes have the CD8 receptor on their cell surface. CD8 positive cells are cytotoxic or suppressor in function. When chicken lymphocytes are treated with anti-CD8 antibody and complement cytolytic activity induced toward allogeneic or

reticuloendotheliosis virus transformed target cells is reduced (Chan *et al.* 1988, Lillehoj *et al.* 1988).

The functional classification of T cells by CD markers may not be absolute. There are examples of $CD4^+$ cells that are cytotoxic and $CD8^+$ cells that secrete IL-2 *in vitro* (Bierer *et al.* 1989). Apparently CD4 or CD8 participates in antigen recognition by increasing the affinity that the T cell antigen receptor has for presented antigen. The main immune function of CD4 and CD8 molecules may be to serve as co-recognition markers by directly interacting with class I or class II, respectively, on the APC (Bierer *et al.* 1989).

The $CD8^+$ T cells recognize antigen presented in association with class I MHC molecules through the antigen specific T cell receptor (Tcr) and physically bind to the APC. The $CD4^+$ T cells bind to APC that express antigen in association with autologous class II MHC molecules. The binding of the T cells induces the macrophage to secrete IL-1. The IL-1 in turn results in the clonal expansion and differentiation of the T cells that have bound to the presented antigen and are now referred to as activated cells. Antigen presentation to the $CD4^+$ T cells results in the increased synthesis of IL-2 and IL-2 receptor by these cells. The IL-2 results in additional proliferation of antigen-specific $CD4^+$ and $CD8^+$ cells.

The activated $CD8^+$ T cells expand in number and differentiate into functional cells. Some activated $CD8^+$ T cells are functionally cytotoxic and will destroy cells bearing autologous class I MHC molecules and the antigen fragment their Tcr recognizes. These cells especially contribute

to immune surveillance of virus- or cancer-transformed cells (Nabholz and MacDonald 1983). $CD8^+$ T cells are also involved in antigen-specific suppressor activity through mechanisms that are not well defined or widely accepted (Green *et al.* 1983).

Simultaneously with T cell activation, B cell activation is occurring. The B cells interact with free antigen through their antigen-specific receptor (surface bound immunoglobulin). The antigen is bound by the immunoglobulin and engulfed by the cell. The endocytosed complex is digested, and subsequently, pieces of the antigen are expressed on the cell surface in association with class II MHC molecules. The activated helper T cells ($CD4^+$) that can recognize the presented antigen (through their Tcr) bind to the B cells. The bound T cells in turn release IL-4 which starts the clonal expansion and differentiation of the B cells into immunoglobulin-secreting plasma cells (Paul and Ohara 1987). At least two other interleukins (IL-5 and IL-6) contribute to the complete development of the plasma cells (Kishimoto and Hirano 1988).

A great amount of chicken T cell functional characterization took place before the development of antibodies capable of defining T lymphocyte subsets (for a review see: Chi and Thorbecke 1987). Monoclonal antibodies that can identify avian CD4 and CD8 have recently been developed by two research groups (Chan *et al.* 1988, Lillehoj *et al.* 1988). The availability of these monoclonal antibodies has made it possible to explore the nature of cells involved in antigen-specific reactions in chickens.

The Nature of Immunologic Non-response

It is possible that the humoral immune response to GAT is mediated at some other level of cell interaction or by cell surface proteins other than Ia (B-L). For example, the T cell's contributions to immune response or antigen presentation (both subject to MHC restriction) could be determining factors. There is evidence for non-response to antigen (measured as antibody production) being primarily a T-cell mediated phenomena (Janeway 1983) and mechanisms of disease resistance in chickens, though poorly understood, require T lymphocyte involvement (Lillehoj 1991).

Janeway (1983) proposed three generalized hypotheses to account for non-response to antigen:

1. Failure of the antigen to interact with Ia molecule (really an antigen presentation defect).

This is usually due to MHC haplotype differences. There are some *I* region haplotypes that fail to interact with antigen.

2. Absence of T cells (or particular T cell subpopulations) that can interact with the Ia-antigen complex on the APC.

Absence of reactive T lymphocytes could be due to the maturation process in the thymus (auto-reactive thymocytes are clonally eliminated) or the existence of natural tolerance (Von Boemer *et al.* 1978).

3. Induction of antigen-specific suppressor T cells.

In the mouse, low or non-response to GAT is mediated through the T cells and is seen as a dominance of suppression over help for immune cells (Gershon *et al.* 1983, Pierce *et al.* 1988). Pierce *et al.* were able to

demonstrate the induction of T-cell populations that were specific for the antigen (GAT) and had the suppressor phenotypic marker (CD8). Antigen-specific activation of suppressor T cell populations does not universally mediate low response. It has not been possible to demonstrate suppressor T cell involvement in mice that are non-responders to pigeon cytochrome c (Schwartz *et al.* 1985).

There are examples that indicate that non-responsiveness is not always due to T cell deficiencies (Herber-Katz *et al.* 1983, Adorini and Doria 1981). Janeway favored the hypothesis that non-response was due to antigen processing defects, but only if induction of antigen-specific suppressor cells could not be demonstrated.

Adorini and Doria (1983) found that "Biozzi" mice, genetically selected for low antibody response to sheep red blood cells, were also low responders to secondary challenge with hen egg white lysozyme (HEL), as measured by T cell proliferation *in vitro*. However, when the secondary HEL stimulation *in vitro* was increased in concentration 50 times, the low responder cells proliferated to a degree similar to high responder cells. This suggested that low responder cells had HEL antigen receptor and that antigen presentation was impaired or defective in some way. It was proposed that the catabolic activity of the low responder macrophages overprocessed the HEL and subsequently expressed antigen on the macrophage surface that was not recognizable by the T cells. The 50-fold increase of antigen *in vitro* overwhelmed the macrophages and resulted in the expression of a less processed antigen that was capable of stimulating the T cells. Mouton *et al.* (1984), using the same mouse lines, found similar

phagocytic activity when they compared high and low responder sublines by carbon clearance assay. They also found evidence that antigen persisted longer in high responder mice. The antigen turn-over rate was faster in low responder mice and indirectly indicated faster catabolism. It was possible that the antigen was being removed before immune cell activation occurred.

The ability to produce antibody is the end result of a chain of reactions involving different cells and soluble mediators. The humoral immune response to GAT has been linked to important immunological traits as well as disease resistance, but the underlying mechanisms are poorly understood. The purpose of the research presented in this dissertation was to investigate some of the reactions, presumably mediated through different immune effector cells, that contribute to antibody production to GAT in the S1 line of chickens. Most of the reactions were studied *in vitro* to dissect the appropriate cells that participate in the immune reaction.

MATERIALS AND METHODS

Experimental Animals

All chickens used in these studies were produced and housed at the Iowa State University Poultry Research Center. Chickens were housed in conventional floor pens or wire cages and received food and water *ad libitum*. All chickens received vaccinations for Marek's disease and fowl pox. The S1 line chickens (described in the Literature Review) are maintained from generation to generation by mating five males of each subline to approximately five hens (per male) of the same subline by artificial insemination. Breeders were selected to represent the appropriate *Ea-B* and *Ir-GAT* types. Ten to 20% of the progeny in each generation were selected as breeders. The inbreeding coefficients of these sublines averaged .40 in 1985 (Cheng *et al.* 1985).

Chickens of the highly inbred (greater than 95%, Knudtson 1987) GHs line were used in some phases of research. The MHC haplotypes B^6 and B^{13} are maintained as sublines in this line and the sublines can be considered congenic. The GHs line has been widely used in immunogenetic studies and is referred to as G-B1 and G-B2 (B^{13} and B^6 respectively) by other laboratories (Miggiano *et al.* 1976, Pink and Miggiano 1977, Morrow and Abplanalp 1981, Maccubbin and Schierman 1986).

Complementation matings

Two types of crosses were made to test for evidence of gene complementation between low responder sublines. The first cross was

between B^{19} *Ir-GAT*^{L} and B^{1} *Ir-GAT*^{L} chickens of the S1 line. The second type of cross was between S1 line B^{19} *Ir-GAT*^{L} chickens and GHs line B^{13} chickens (also low responders to GAT challenge). Progeny from the crosses were challenged with GAT at 12 weeks of age and the immune sera were assayed by GAT radioimmunoassay (RIA).

Backcrosses to parental sublines and *inter se* matings were made with the GHs X S1 F_1 chickens produced. These progeny were MHC typed (*B-F/B-G*) by hemagglutination at six weeks and challenged for GAT response at twelve weeks of age.

GAT Immunization Challenge

The random linear amino acid polymer GAT (L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}) was obtained from Miles Scientific (Naperville, IL). All work was completed with material from the same lot number (3/19). On day one of the protocol, each chicken (12 to 16 weeks of age) was intramuscularly immunized with 1 ml total volume of antigen emulsion. The emulsion was 1 part complete Freund's adjuvant (Gibco Laboratories, Grand Island, NY): 1 part GAT in phosphate buffered saline (PBS: 0.5 M phosphate, 0.85% NaCl, pH 7.2), 0.5 ml in each side of the breast. Total dose of antigen was 250 μg. On day 21 a second dose (0.5 ml of 500 μg GAT/ml in PBS pH 7.2) was administered intravenously. The chickens were bled on day 14 (primary response) and day 28 (secondary response) and the sera were collected and frozen until assayed.

Radiolabelling of GAT

GAT was labelled with ^{125}I ($Na^{125}I$, IMS.300, Amersham Corporation, Arlington Heights, IL) by the chloramine-T method (Hunter 1973). Briefly, 250 μg GAT in 0.5 ml of phosphate buffer (10 mM $NaH_2PO_4 \cdot H_2O$, 40 mM $Na_2HPO_4 \cdot 7H_2O$ pH 7.5) was placed in a 5-ml beaker on ice on a magnetic shaker. One mCi of ^{125}I (IMS.300 is packaged as virtually no volume, PBS was added to the vial to facilitate removing 1 mCi) was added to the GAT solution, followed by 0.1 ml of chloramine-T solution (7 mM chloramine-T in phosphate buffer, pH 7.5). The reaction was allowed to proceed with stirring for five minutes. After 5 minutes, 0.1 ml of sodium meta-bisulfite solution (10.5 mM $Na_2S_2O_5$ in phosphate buffer, pH 7.5) was added to the reaction mixture. After 3 minutes of additional stirring, 0.1 ml of potassium iodide solution (6 mM KI in phosphate buffer, pH 7.5) was added. Free ^{125}I was separated from the labelled GAT by size exclusion chromatography over a 35 mm X 8 mm Sephadex G-50-150 (Sigma Chemical Company, St Louis, MO) column using a phosphate buffer mobile phase. One ml fractions were collected and 20 μl aliquots from each were counted on a gamma counter. The first peak was the ^{125}I labelled GAT and the second peak was free ^{125}I. The labelled fraction with the highest counts per minute was kept as frozen aliquots and used for the radioimmunoassays within one month of labelling.

GAT radioimmunoassay

A modified Farr radioimmunoassay (Bluestein *et al.* 1971) was used to determine antigen binding by immune serum. The labelled GAT was diluted

1:1000 in assay buffer (PBS pH 7.2 with 1% normal chicken serum). Antisera were diluted 1:10 in assay buffer. Normal chicken serum (NCS) was diluted and used for positive and negative controls. For the assay, 25 μl of the labelled GAT dilution was mixed with 25 μl of the antiserum dilution in a 1.5 ml microcentrifuge tube. The primary reaction was allowed to incubate for one hour at 4° C. After the incubation, 50 μl of rabbit anti-chicken IgG (prepared in-house) was added to the samples and the negative control (NCS) tubes. Positive control tubes (antigen plus NCS) received 50 μl of 20% trichloroacetic acid (TCA). TCA precipitates all the protein, including the labelled GAT, but free ^{125}I remains in the supernatant. The samples were incubated for an additional hour at 4° C. Following the second incubation, the samples were centrifuged (10 minutes in a microcentrifuge) and a 50 μl sample of supernatant was removed for assay on a gamma counter. Results were expressed as the percentage labelled antigen bound by the immune serum dilution using the following formula:

$$\% \text{ Bound} = \left[100 - \frac{(\text{Exp. sample count} - \text{TCA sample count})}{(\text{NCS sample count} - \text{TCA sample count})}\right] X100$$

Protocol to Raise and Test B-L Allo-antisera

The immunization schedule used by Crone *et al.* (1981) to produce B-L allo-antisera was used in producing anti-B-L antibody in the S1 line through reciprocal immunizations between GAT high and GAT low responder chickens. A pre-immune sample was collected from all recipients (16-20 week old chickens) before immunizations were started. On day one of the schedule, all recipients received 8 X 10^8 splenic lymphocytes intra-

venously in 0.5 ml of phosphate buffered saline (PBS: 0.5 M phosphate, 0.85% NaCl, pH 7.2). These cells were administered on the same day as collected from adult S1 chickens and were isolated by centrifugation over Histopaque 1077 (Sigma) density gradient. Additional doses (5 X 10^8) of fixed cells [isolated cells were fixed in 0.25% glutaraldehyde in PBS for 10 minutes, washed three times in PBS with 5% fetal calf serum (FCS, Sigma), and stored at 4° C in PBS with 5% FCS and 0.1% NaN_3] were administered on days 7, 14, and 21. Immune sera were collected on days 9, 17, 24, and 28 and were frozen until assayed, which was within six months.

Radiolabelling of cells

The antibodies were tested for reactivity by several methods. The first type was by immunoprecipitation of radiolabelled B-L positive cells. The cells were labelled one of two ways. Radiolabelling with $Na^{125}I$ (Amersham) was carried out by lactoperoxidase-catalyzed reaction (Lambris and Papamichail 1980, Vitetta *et al.* 1971). Briefly, isolated splenic lymphocytes (1-2 X 10^8) were suspended in 1 ml of PBS containing 1 mCi of ^{125}I and 200 μg of lactoperoxidase (Sigma). The reaction was started by the addition of 50 μl of a 0.03% solution of H_2O_2. Three additional aliquots of H_2O_2 were added at 2½ minute intervals. The reaction was stopped by the addition of 10 ml of cold (4° C) PBS. After four washes in PBS, the cells were ready for lysis to isolate cell membranes.

Alternatively, some cells were biosynthetically labelled with ^{35}S-methionine in a procedure adapted from Guillemot *et al.* (1986) and Charron *et al.* (1983). Splenic lymphocytes were aseptically collected, washed

three times, and placed in culture in Dulbecco's modified Eagle's medium without methionine (DMEM, Gibco Laboratories, Grand Island, NY) supplemented with 10% dialyzed (against PBS) FCS, 300 μg/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all supplements from Sigma). The cultures were incubated for one hour and then pulsed with 250 μCi of ^{35}S-methionine (Amersham) for four to five hours in a 41° C under 5% CO_2 in a humidified incubator. Labeling was stopped by the addition of cold (4° C) PBS. The cells were washed three times in PBS and were ready for preparation of membrane lysates.

Preparation of cell membrane lysates

Cell membrane lysates were prepared as follows. ^{125}I-labelled cells were lysed in a buffer described by Bumstead and Curtis (1986). First, the labelled lymphocytes were centrifuged and the supernatant removed. The pellet of cells was resuspended in 1.0 ml of NP-40/deoxycholate lysis buffer (0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.01% phenylmethylsulfonylfluoride in PBS pH 7.2). The suspension was gently shaken for one hour at room temperature. After lysis had occurred, the unsolubilized material was separated by centrifugation (10,000 X g for 10 minutes). The supernatant was removed and frozen (-4° C) until analyzed by immunoprecipitation and gradient polyacrylamide gel electrophoresis (PAGE) within two weeks.

Cells labelled biosynthetically were lysed in Tris-buffered saline (50 mM Tris pH 7.0, 150 mM NaCl, and 0.02% NaN_3) containing 0.5% NP-40. The cells were incubated in the Tris lysis buffer for 15 minutes at 4°C

and then centrifuged for 30 minutes (10,000 X g, 4°C). The supernatant was collected and used immediately for immunoprecipitations.

Immunoprecipitation reaction

Cell lysates were pre-cleared by incubation with either formalin fixed *Staphylococcus aureus* cells or protein A-coated Sepharose beads (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) to remove material that would react non-specifically prior to the immunoprecipitation reaction. The membrane lysate from 1 X 10^7 cells was reacted with 100-200 μl of antiserum for 4-8 hours at 4°C on a shaker. This reaction formed the primary immune complex which was then precipitated by the addition of rabbit anti-chicken IgG and protein A-Sepharose. The primary immune complex was bound to the Sepharose and collected by centrifugation (10 minutes, 10,000 X g). The complex-Sepharose was washed three times in NP-40/deoxycholate lysis buffer and eluted by boiling for 10 minutes in 50-100 μl sample buffer (0.125 M Tris pH 6.8, 4% sodium dodecylsulfate (SDS), 20% glycerol, 0.1 M dithiothreitol, and 0.05% bromophenol blue). The eluted complex was then ready for electrophoresis on gradient gels.

Polyacrylamide gel electrophoresis

Gradient polyacrylamide slab gels were prepared as describe by Hames (1981) using the concentrations described by Laemmli (1970). The gradient ran from 5% to 15% acrylamide with a 3.75% discontinuous stacking gel. Samples (10-40 μl) were added to the wells and electrophoresed at 25 mA constant current until the tracking dye reached the bottom of the gel

(usually 2.5 to 3 hours). After removal from the gel cassette, the gels were dried onto a piece of filter paper on a gel drier. The dried gels were used to expose x-ray film for autoradiograms.

Western blot analysis of antisera

Antisera were also assayed by Western or immuno-blotting using the procedures reported by Bumstead and Curtis (1986). Briefly, unlabelled cell lysates (prepared the same as labelled cell lysates) were prepared for electrophoresis by boiling 10-25 μl of lysate with an equal volume of sample buffer (0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 0.1 M dithiothreitol, 0.05% bromophenol blue) for 10 minutes. The sample was then transferred to the well of a polyacrylamide gel and electrophoresed. After electrophoresis, the gel was removed and the separated proteins were transferred to nitrocellulose by electro-transfer (30 V, constant, for 8-12 hours). After transfer, the open reactive sites on the nitrocellulose were blocked by incubating the blot in PBS-Tween (PBS pH 7.2 with 5% Tween-20) for at least 1 hour at room temperature. The blot was then incubated with the primary chicken antibody (used at dilutions of 1:50 to 1:100) in PBS-Tween for at least 4 hours at room temperature. After the first incubation, the blot was washed in PBS-Tween and incubated with the second antibody [biotinylated-rabbit anti-chicken IgG (Zymed Laboratories, South San Francisco, CA)], diluted 1:1000 in PBS-Tween. After a 2-3 hour incubation at room temperature, the blot was again washed and a 1:2500 dilution of streptavidin-alkaline phosphatase (Zymed) was added to the blot for 2 hours at room temperature. After the last incubation, the blot

was washed in PBS-Tween and BCIP/NBT substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium chloride, available as a kit from Zymed) was added and the color reaction was allowed to develop. Development was stopped by washing the blot in distilled water.

Antigen-induced *In Vitro* Proliferation Assay

In vitro proliferation of T lymphocytes induced by GAT was assayed by a procedure modified from Vainio *et al.* (1988). Blood was collected by heparinized syringe from the jugular vein of chickens primed by GAT immunization (250 μg GAT in 0.5 ml PBS emulsified with 0.5 ml of complete Freund's adjuvant-i.m.) two weeks previously. Peripheral blood lymphocytes (PBL) were separated from whole blood by centrifugation over Histopaque 1077 (Sigma) density gradient. The lymphocyte fraction was collected aseptically and washed three times with culture medium (RPMI 1640 with 25 mM HEPES (Gibco) supplemented with 100 U/ml penicillin, 100 μg/ ml streptomycin, 5% of heat-inactivated normal chicken serum (Sigma) and 5 X 10^5 M 2-mercaptoethanol). After the last wash, the cells were resuspended in the medium, counted, and the cell concentration was adjusted to 1 X 10^7 cells/ml.

Cell suspensions were added to sterile, flat bottom, 96-well tissue culture plates (Flow Laboratories, McLean, VA) (100 μl/well containing 10^6 cells). An additional 100 μl of medium with or without antigen was added to each well. Final GAT concentrations of 0, 1, or 10 μg/well were routinely used. Cells from each chicken were cultured with all three concentrations of GAT in quadruplicate. After the cultures were set up, they

were incubated in a humidified, 41° C, 5% CO_2 incubator for three days. After three days, proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

MTT *In Vitro* Proliferation Assay

T cell proliferation *in vitro* was measured (as viable cells) by a colorimetric assay based on cleavage of MTT by living cells (Mosmann 1983). Stock solution of MTT (10 mg/ml) was prepared in PBS, filtered (0.45 μm), and stored in dark glass at 4° C until used (within two weeks). At the end of the culture period, 20 μl of MTT stock solution was added to each well of the plate. The plate was returned to the incubator for an additional 3 hours. At the end of incubation, 150 μl of medium was removed, without disturbing the cells, from each well and replaced with 150 μl of acid-isopropanol (3.3 ml 12 N HCl/liter of isopropanol). The plate was then vortexed to insure lysis of cells and to dissolve the cleaved MTT. Absorbance at 570 nm was determined for each well on an ELISA plate reader (Model ELIA Reader, Fisher Scientific Co., Itasca, IL).

In Vitro Antigen-presenting Cell Assay

Plastic-adherent cells from Histopaque 1077 (Sigma) isolated PBL were used as antigen-presenting cells (APC) for *in vitro* stimulation of *in vivo*-primed T cells. The adherent mononuclear cells are primarily monocyte/macrophage cells (Chu and Dietert 1989) and were prepared by a method used by Vainio *et al.* (1988). PBL were first isolated from whole blood of GAT primed-chickens by gradient centrifugation over Histopaque

1077. The mononuclear cell layer at the interface was collected and washed three times in supplemented RPMI 1640 (see Antigen-induced *In Vitro* Proliferation Assay); the cell concentration was adjusted to 5 X 10^6 cells/ml. The cell dilutions were plated into a 96-well plate (200 μl containing 10^6 cells/well). The cultures were placed in an incubator (humidified, 41° C, 5% CO_2) for adherence to occur (1-2 hours). After the first incubation, the non-adherent cells were washed away (3X) with fresh, prewarmed (41° C) medium. Medium with antigen (GAT at 10 μg/well in 200 μl) was then added to the remaining adherent cells. Negative controls received 200 μl of medium without antigen. The cultures were returned to the incubator for a 5 hour antigen pulse. During the antigen pulse, the responder T lymphocytes were prepared.

Responder cells from GAT-primed chickens were isolated from whole blood by centrifugation over Histopaque 1077. Isolated cells were washed three times in medium and the cell concentration was adjusted to 5 X 10^6 cells/ml in supplemented medium. After the APC culture had been antigen pulsed for five hours, the adherent cells were washed three times with medium (no GAT) to remove the non-internalized GAT and the prepared responder cell dilutions (200 μl volume) were added to them without any additional antigen. The cultures were returned to the incubator for a three-day incubation before proliferation was determined by MTT assay. Responder cells and APC were combined within *Ea-B* type (to avoid mixed lymphocyte proliferative reaction) and were matched or mismatched by *Ir-GAT* type.

Fluorescent Labelling of Cultured Cells for Flow Cytometry

Cell surface phenotype of cells receiving secondary exposure to GAT *in vitro* was determined by fluorescent antibody labelling and analysis by flow cytometry. Cells cultured (as described in Antigen-induced *in vitro* Proliferation Assay) with 0, 1, or 10 μg/well concentration of GAT for three days were collected for analysis. Non-adherent cultured cells were placed in siliconized 12X75 mm glass tubes and washed once in cold (4° C) wash buffer (1.1X Dulbecco's PBS (Sigma) plus 2% heat-inactivated fetal calf serum, plus 0.1% NaN_3) before labelling.

Monoclonal antibodies (as hybridoma culture supernatants) were obtained from two sources. Anti-CD8 was a gift from Dr. Hyun Lillehoj (USDA-ARS, Beltsville, MD) and was used at a dilution of 1:10 (in wash buffer). The anti-CD8 is a mouse monoclonal antibody which identifies chicken T lymphocytes of the cytotoxic/suppressor functional type (Lillehoj *et al.* 1988). An anti-CD4 monoclonal antibody (culture supernatant) was a gift from Dr. Chen-lo Chen (University of Alabama, Birmingham, AL). The anti-CD4 antibody identifies a molecule that is the avian homologue of the surface marker on T cells of the helper phenotype (Chan *et al.* 1988). Anti-CD4 antibody was used at a dilution of 1:10.

After one wash, the harvested cultured cells were resuspended in 100 μl of primary antibody dilution (anti-CD4 or anti-CD8) or wash buffer only (for controls). The cells were incubated for 30 minutes at 4° C. Following incubation, the cells were washed three times with cold wash buffer and the second antibody was added.

The second antibody was fluoroisothiocyanate (FITC)-conjugated

rabbit anti-mouse IgG $F(ab')_2$ fragments (Sigma) diluted 1:100 with wash buffer. Washed cells were resuspended in 100 μl of the second antibody dilution and incubated for 30 minutes at 4° C. Controls received no primary or secondary antibody or secondary antibody only.

Following the second incubation, the cells were washed three times with cold wash buffer. After the last wash, the cells were fixed by first resuspending the cells in 200 μl Dulbecco's PBS (with 0.1% NaN_3) and then adding 200 μl of 2% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) solution (in DPBS + 0.1% NaN_3). Samples were covered with aluminum foil and refrigerated (4° C) until analyzed by flow cytometry.

Flow cytometry

Labelled cells were analyzed on an EPICS Profile I flow cytometer (Coulter Corporation, Hialeah, FL). Lymphocyte populations were selected for fluorescence analysis based on forward light scatter versus side light scatter (size versus granularity) histograms. The analyzed lymphocyte populations represented an average of 84% of the cultured cells through the flow cytometer. The flow cytometer was electronically gated to exclude background fluorescence from analysis. Results were recorded as percent fluorescence positive cells (within gated fluorescence window) of 4 X 10^3 cells tested.

Statistical Analysis

Analyses of variance were performed on assay results using the values from different assays as the variables and the traits of selection

(Ea-B, *Ir-GAT*) as sources of variation. Differences between means were tested by Duncan's multiple range test or by *t* test (SAS Institute Inc. 1985).

RESULTS

Anti-B-L Alloantisera

Despite the immunization of hundreds of chickens (84 by one protocol alone), none of the immunization protocols resulted in an antiserum that could discriminate between Ir-GAT^{H} and Ir-GAT^{L} chickens. None of the screening methods used, despite their sensitivity in detecting B-G or B-F antigens, could detect *B-L* allo-antibody in serum from chickens used in reciprocal immunizations. Figure 2 shows the results of a Western (immuno) blot assay using anti-1L antiserum as the primary antibody versus bursal lymphocyte membrane lysates of each of the four S1 haplotypes. The bands above 50 kd are due to cross reactivity with the streptavidin-phosphatase conjugate used to detect the primary antibody. The bands in lanes 1 and 2 are the B-F protein.

B-G antigen was also detectable by immunoblot assay. Figure 3 shows the results of probing B^{6} and B^{13} red blood cell (RBC) membrane lysates with anti-B^{13} antiserum. Arrows indicate the B-G13 protein from the B^{13} RBC lysate. The double bands in the lanes where the sample was not reduced may be due to partially reduced proteins migrating separately or variation in the length of the cytoplasmic portion of the molecule under non-reduced conditions.

GAT-induced *in vitro* Proliferation of Antigen-primed Lymphocytes

Two sets of chickens were used to determine if there were measurable differences in proliferation when lymphocytes from GAT-stimulated chickens

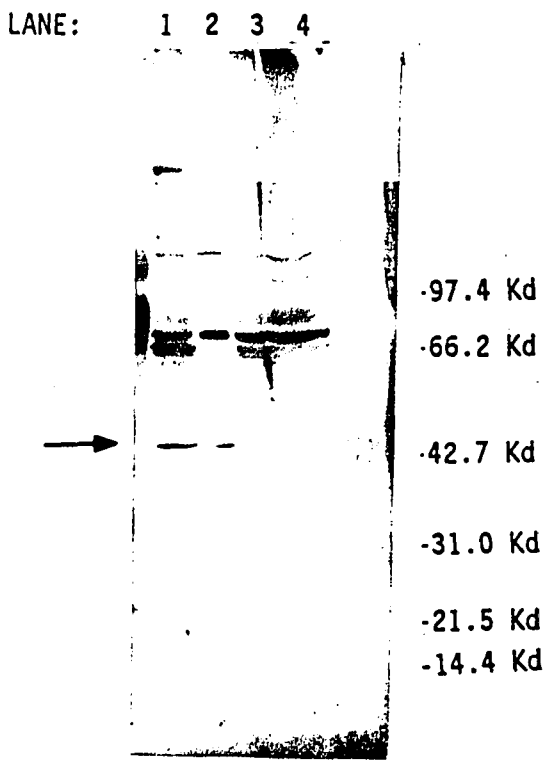


Figure 2. Western (Immuno)-blot of bursal lymphocyte membrane proteins separated by gradient polyacrylamide gel electrophoresis. Primary antiserum was produced by immunizing S1-19H chickens with splenic lymphocytes from S1-IL chickens. Lane 1, 1H lysate; lane 2, 1L lysate; lane 3, 19H lysate; lane 4, 19L lysate. Arrow indicates B-F band

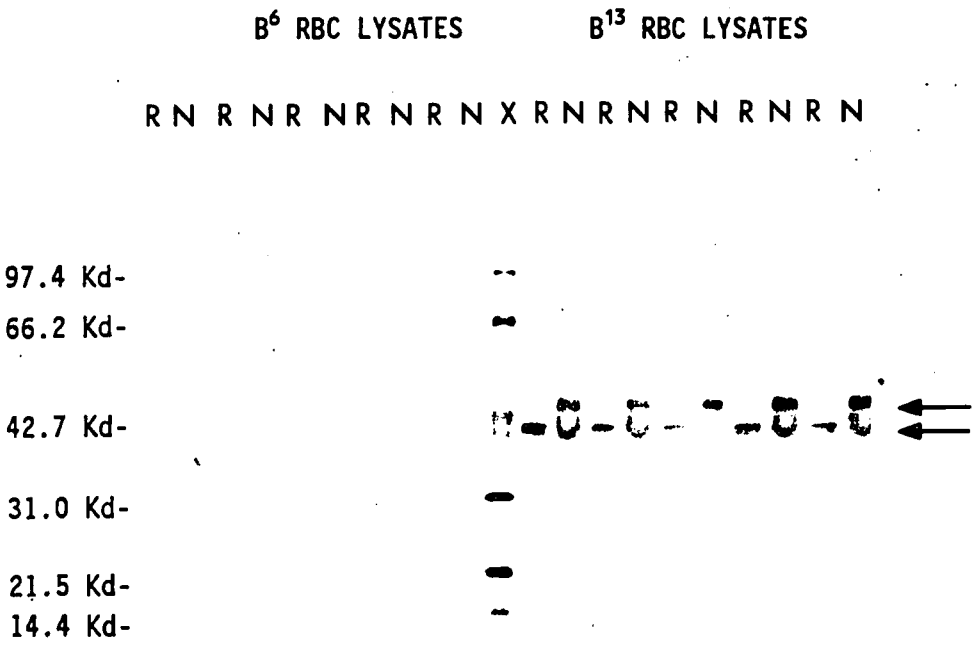


Figure 3. Western (Immuno)-blot of GHs line red blood cell (RBC) membrane proteins separated by gradient polyacrylamide gel electrophoresis. Primary antiserum was produced by immunizing GHs-6 chickens with GHs-13 RBC. Lane marked X contains molecular size standards. Lanes marked R or N contain lysates that were reduced (R) or non-reduced (N). Arrows indicate B-G bands

received a second stimulation *in vitro*. The first assay group were all hens and were at least one year of age. The second group of birds was of both sexes assayed between twelve and sixteen weeks of age.

Table 2 shows the results for the first assay group (shown graphically in Figure 4). There was no difference in amount of proliferation within a haplotype among the concentrations of GAT. There were differences between haplotypes within a level of GAT (Table 3). In general, the cells from Ir-GAT^{H} chickens proliferated more than cells from the Ir-GAT^{L}. Table 4 shows the analysis of variance on absorbance at 570 nm at each concentration of GAT used in the culture medium. At each level of GAT, the Ir-GAT type of the cells was a significant source of variation and blood group (Ea-B type) was not significant.

Table 2. Mean MTT values (absorbance at 570 nm) ± standard error for cultured cells from the first assay group of chickens. Lymphocytes were assayed at three levels of GAT in the medium. Means within a column not sharing a letter are significantly different ($P<0.05$)

	GAT CONCENTRATION IN MEDIUM					
	0 GAT		1 μg GAT		10 μg GAT	
MHC TYPE	N[a]	MTT ASSAY A^{570}	N[a]	MTT ASSAY A^{570}	N[a]	MTT ASSAY A^{570}
19H	10	$1.360^{A} \pm .08$	10	$1.289^{A} \pm .08$	10	$1.427^{A} \pm .08$
19L	9	$1.009^{B} \pm .14$	9	$0.965^{B} \pm .11$	9	$1.056^{B} \pm .14$
1H	8	$1.295^{AB} \pm .09$	8	$1.273^{A} \pm .08$	8	$1.419^{A} \pm .09$
1L	8	$1.012^{B} \pm .05$	8	$0.992^{B} \pm .06$	8	$1.062^{B} \pm .06$

[a]Number of chickens assayed.

Table 3. Analysis of variance of MTT values (absorbance at 570 nm) by concentration of GAT in the medium. Cells from chickens of the first assay group

	0 μg GAT/WELL		1 μg GAT/WELL		10 μg GAT/WELL	
Source	df[a]	MS[b]X10^2	df	MSX10^2	df	MSX10^2
Haplotype	3	28.6*	3	27.0*	3	38.7*
Error	31	8.4	31	6.0	31	8.3

[a]Degrees of freedom.
[b]Mean square.
*P<0.05.

Table 4. Analysis of variance by GLM of MTT values (absorbance at 570 nm) by concentration of GAT. Chickens from first assay group

	0 μg GAT/WELL		1 μg GAT/WELL		10 μg GAT/WELL	
SOURCE	df[a]	MS[b]X10^{-3}	df	MSX10^{-4}	df	MSX10^{-6}
BLOOD TYPE	1	2.7	1	2.3	1	8.3
Ir-GAT TYPE	1	805.7*	1	7917.0*	1	1147127.4*
BT*GAT[c]	1	19.1	1	40.0	1	427.6
ERROR	31	84.5	31	604.3	31	833655.4

[a]Degrees of freedom.
[b]Mean square.
[c]Blood type X Ir-GAT type interaction.
*P<0.005.

To better evaluate the lymphocyte's response to secondary GAT exposure *in vitro*, an index was constructed by dividing the mean absorbance of wells with GAT by the mean absorbance of the same cultures without GAT. This

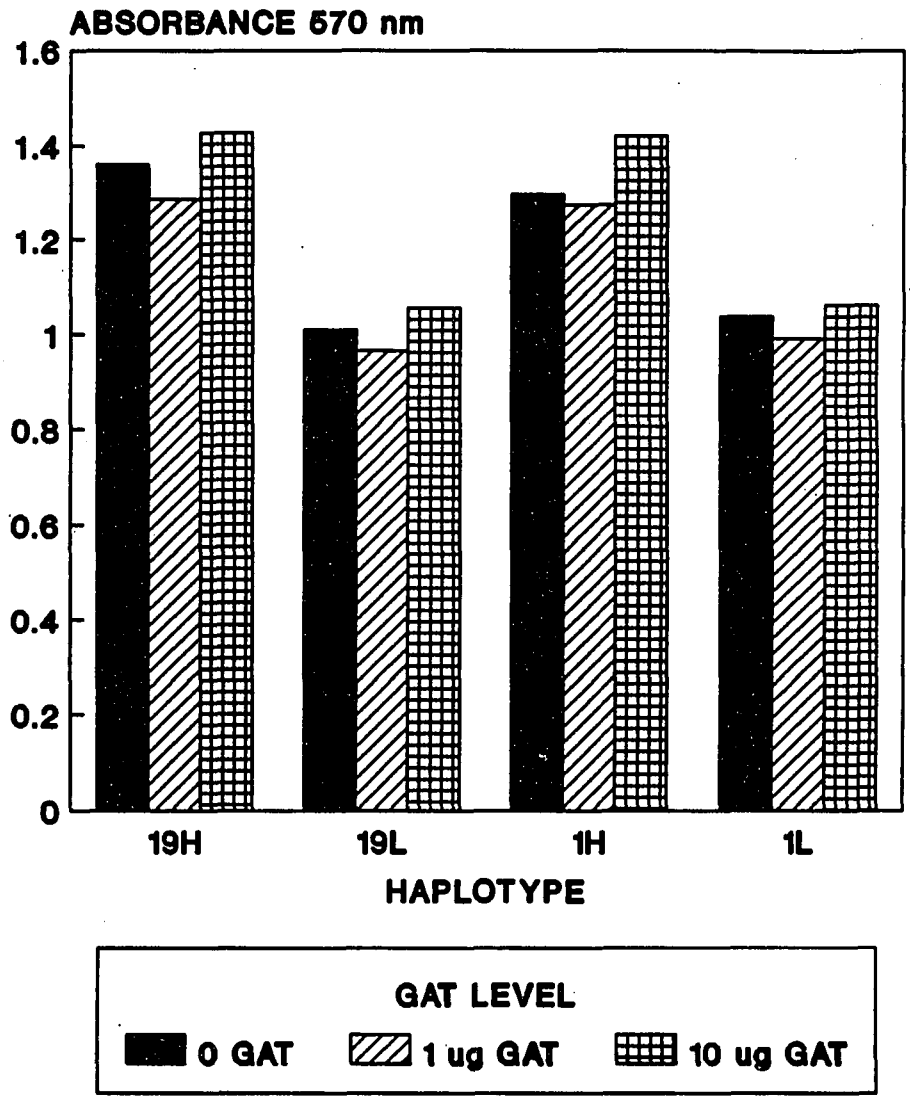


Figure 4. Raw MTT values (absorbance at 570 nm) for the first assay group of chickens. Each haplotype was cultured and assayed at three different concentrations of GAT in the medium

value was multiplied by 100 and the result was subtracted from 100. Thus index scores above 0 indicated percent proliferation above control levels and negative scores indicated percent suppression below control levels. Figure 5 shows the graph of the index scores by haplotype and GAT level (1 or 10 μg/well). Within each haplotype, except 19H, there was a significant difference in the response comparing 1 or 10 μg of GAT. The lower level of GAT suppressed proliferation below control values. Figure 6 shows the results of combining absorbance values by Ir-GAT type. At each level of GAT, the lymphocytes from Ir-GAT^{H} chickens proliferated in culture better than cells from Ir-GAT^{L} chickens.

Table 5 shows the mean absorbance of the second assay group tested at three levels of GAT. Results are shown graphically in Figure 7. Again there were no differences within haplotype between concentrations of GAT. There were differences among haplotypes within a level of GAT (Table 6).

The analysis of variance by GAT concentration in the medium is shown in Table 7. At each level of GAT, the Ea-B (blood) type of the cell donor was a significant source of variation, cells from B^{19} chickens had higher levels of proliferation. At the 10 μg GAT level, Ea-B type and Ir-GAT type of the cells was significant. At the 10 μg level, there was a tendency for the Ir-GAT^{H} cells to proliferate more compared to cells from Ir-GAT^{L} chickens. Response of the cells did not vary by sex of the donor chicken. Figure 8 presents the results of plotting the index score by MHC haplotype. There were no differences in index scores among haplotypes at either level of GAT and the difference between GAT concentrations within haplotype was not significant. The 1 μg level of GAT did not result in

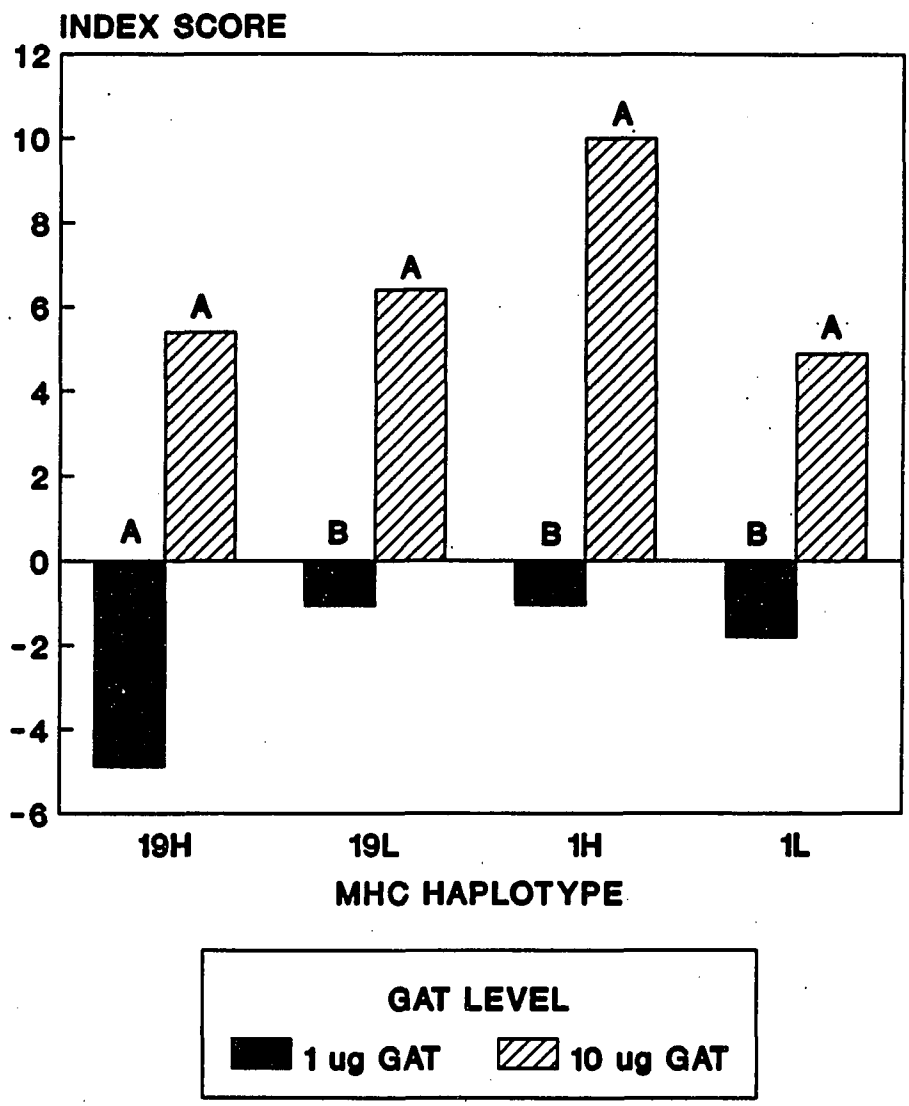


Figure 5. Raw MTT values converted to an index score (percent of control) for chickens of the first assay group. Within a haplotype, columns not sharing a letter are significantly different ($P<0.05$)

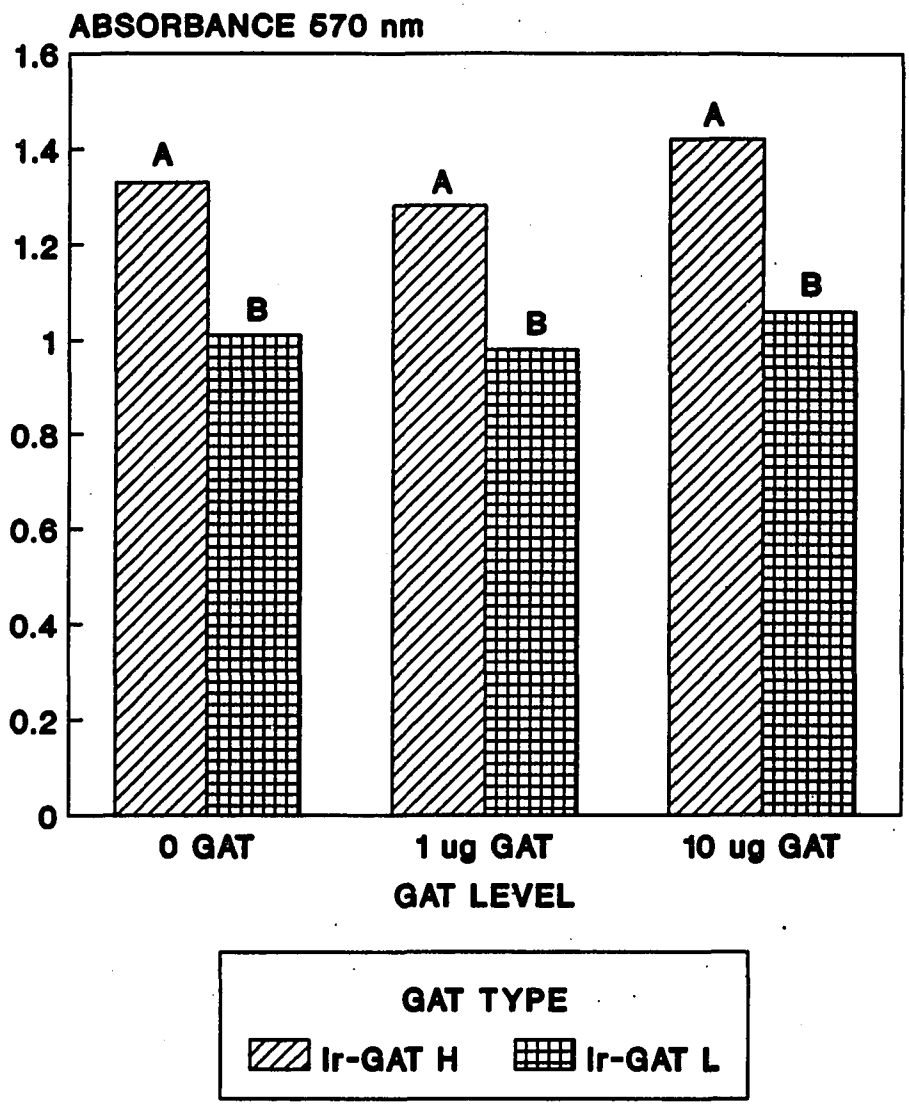


Figure 6. Mean MTT values from the first assay group pooled by Ir-GAT type. Within a level of GAT, columns not sharing a letter are significantly different ($P<0.05$)

Table 5. Mean MTT values (absorbance at 570 nm) ± standard error for cultured cells from the second assay group of chickens. Lymphocytes were assayed at three concentrations of GAT in the medium. Means within a column not sharing a letter are significantly different ($P<0.05$)

	GAT CONCENTRATION IN MEDIUM					
	0 GAT/WELL		1 μg GAT/WELL		10 μg GAT/WELL	
MHC TYPE	N[a]	MTT ASSAY A^{570}	N[a]	MTT ASSAY A^{570}	N[a]	MTT ASSAY A^{570}
19H	16	$0.779^{A} \pm .06$	16	$0.808^{A} \pm .06$	16	$0.836^{A} \pm .06$
19L	16	$0.642^{B} \pm .03$	16	$0.655^{B} \pm .03$	16	$0.683^{B} \pm .03$
1H	16	$0.513^{B} \pm .04$	16	$0.506^{C} \pm .04$	16	$0.569^{BC} \pm .04$
1L	16	$0.541^{B} \pm .05$	16	$0.537^{BC} \pm .04$	16	$0.541^{C} \pm .04$

[a]Number of chickens assayed.

Table 6. Analysis of variance of MTT values (absorbance at 570 nm) by concentration of GAT in the medium. Cells from chickens of the second assay group

	0 μg GAT/WELL		1 μg GAT/WELL		10 μg GAT/WELL	
Source	df[a]	MS[b]X10^2	df	MSX10^2	df	MSX10^2
Haplotype	3	22.9*	3	30.2*	3	28.5*
Error	60	3.4	60	3.3	60	3.2

[a]Degrees of freedom.
[b]Mean square.
*$P<0.001$.

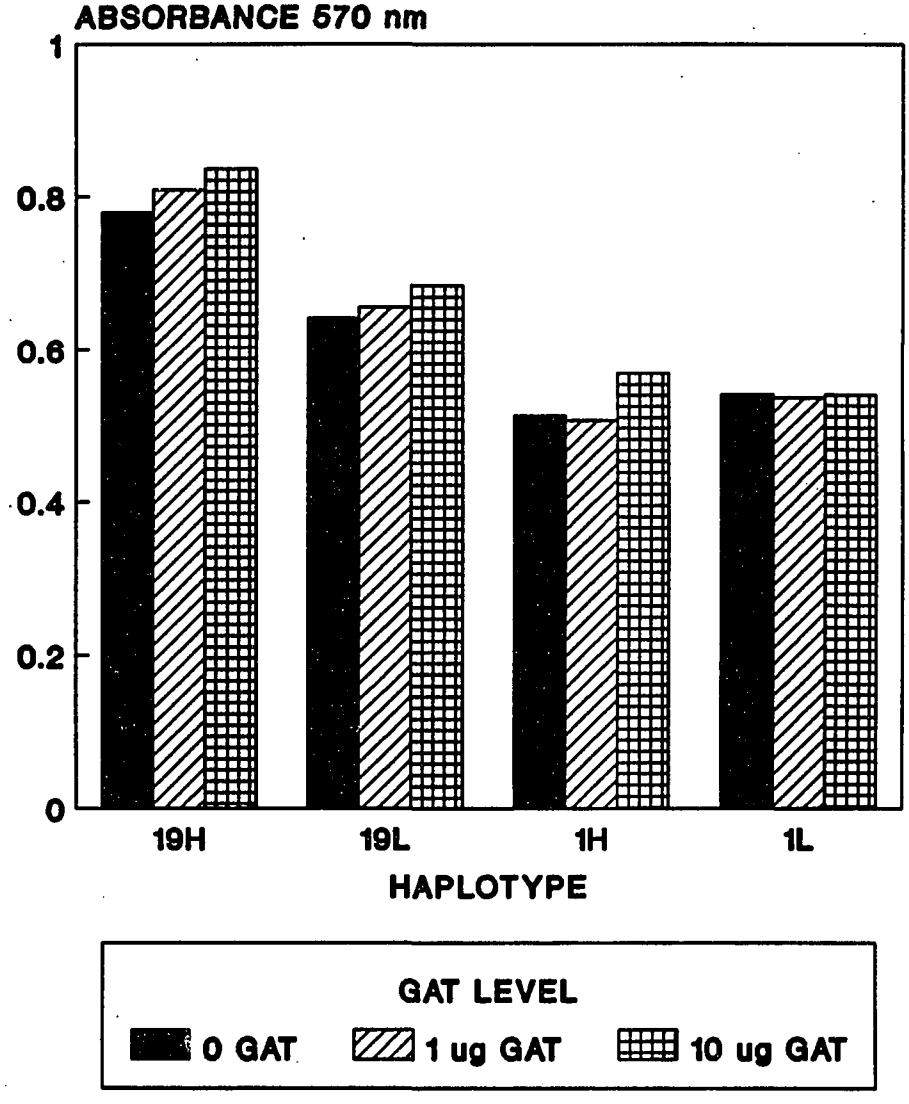


Figure 7. Raw MTT values (absorbance at 570 nm) for the second assay group. Each haplotype was cultured at three concentrations of GAT in the medium

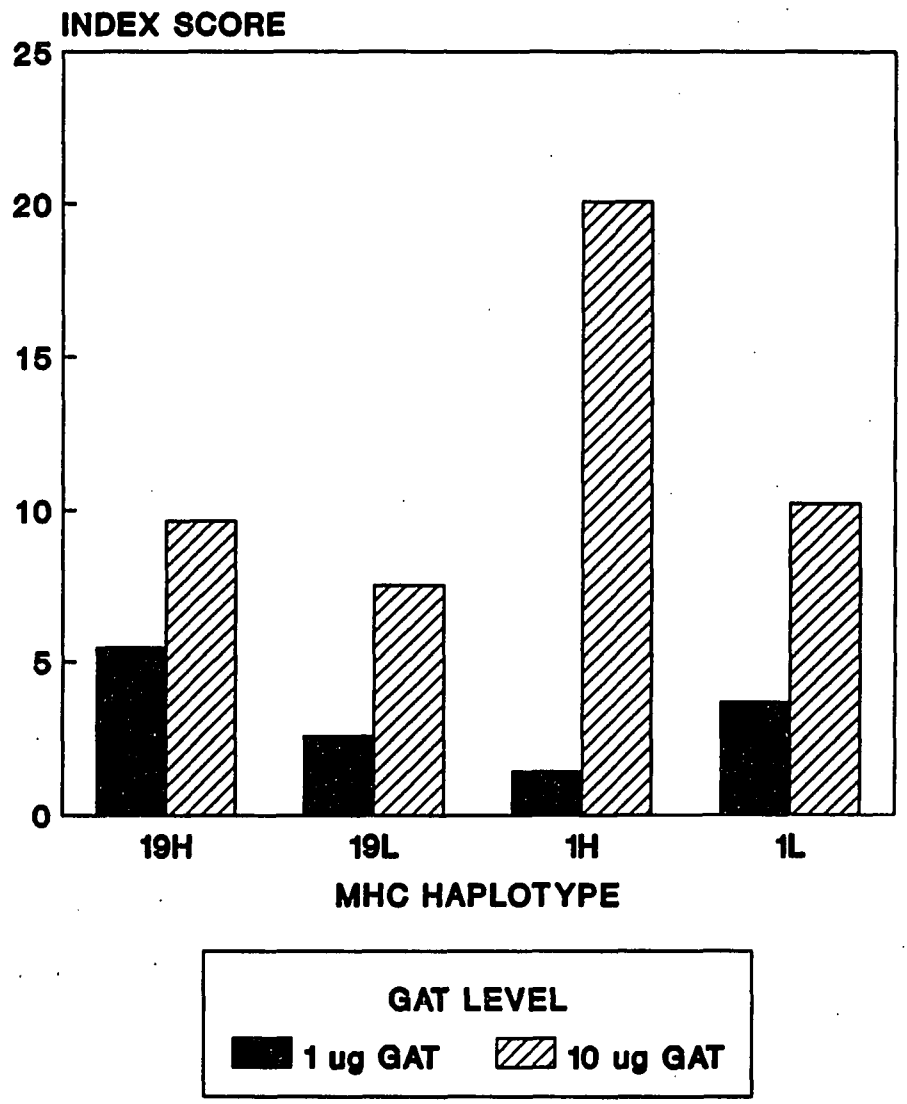


Figure 8. Raw MTT values converted to an index score (percent of control). Second assay group

Table 7. Analysis of variance of MTT values (absorbance at 570 nm) by concentration of GAT for cultured cells from chickens of the second assay group

	0 μg GAT/WELL		1 μg GAT/WELL		10 μg GAT/WELL	
SOURCE	df[a]	MS[b]	df	MS	df	MS
BLOOD TYPE	1	0.53**	1	0.71**	1	0.66**
Ir-GAT TYPE	1	0.05	1	0.06	1	0.13*
BT*Ir-GAT[b]	1	0.11	1	0.14*	1	0.06
ERROR	60	0.03	60	0.03	60	0.03

[a]Degrees of freedom.
[b]Mean square.
[c]Blood type X Ir-GAT type interaction.
*P<0.05.
**P<0.0001.

suppression of proliferation below control levels in this assay group. Figure 9 shows the results of pooling absorbance values within Ea-B type. At each level of GAT, the B^{19} cells proliferated more than cells from B^{1} chickens.

Antigen Presenting Cell Assay

The antigen presenting cells (APC) from GAT primed chickens (all hens, at least one year of age) were used to set up cultures that mixed or matched Ir-GAT type of antigen presenting cells with Ir-GAT type of responder lymphocytes. The APC were pulsed at a GAT level of 10 μg/well and compared to control wells of APC that were not exposed to GAT. Assays were conducted within Ea-B type to avoid mixed lymphocyte reactions.

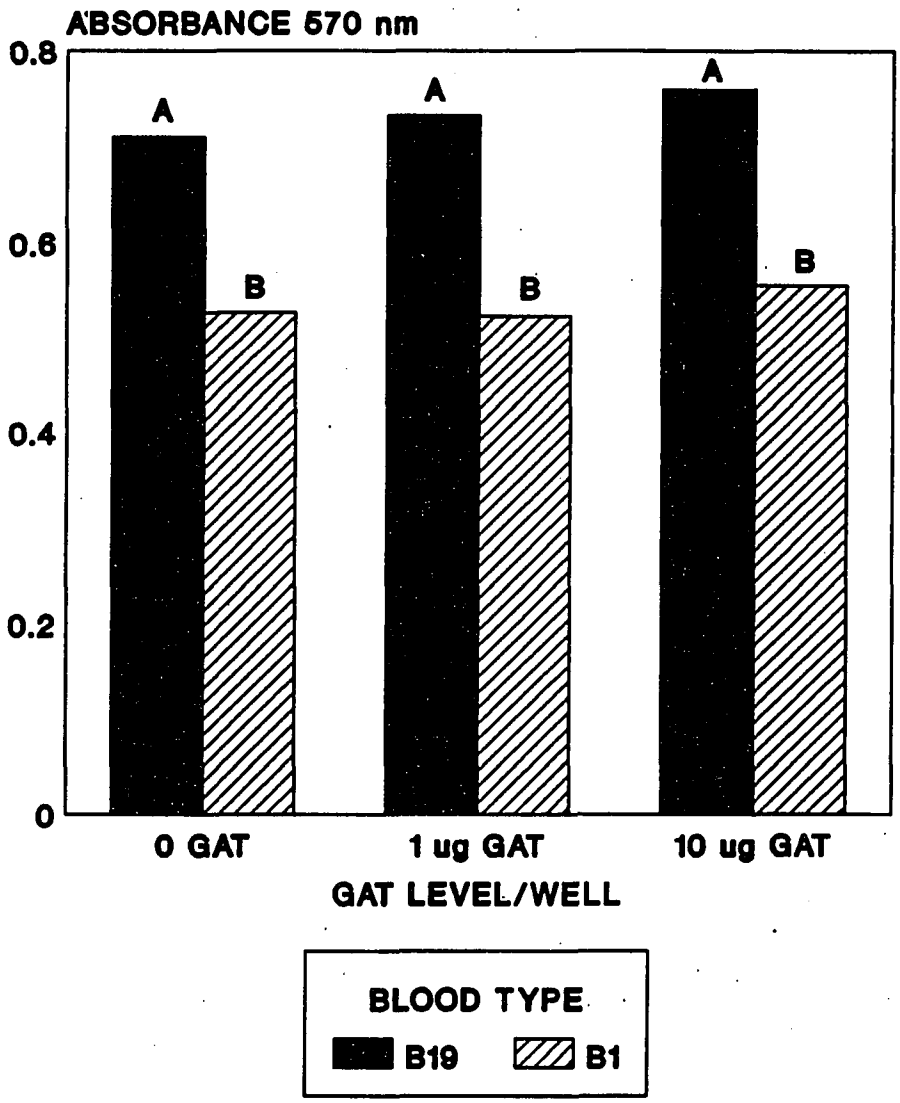


Figure 9. Mean MTT values from the second assay group pooled by blood type. Within a level of GAT, columns not sharing a letter represent means that are significantly different ($P<0.05$)

Table 8. Mean MTT index scores ± standard error for the eight different combinations of antigen presenting cells (APC) with responder cells

APC TYPE	RESPONDER TYPE	N[a]	MEAN MTT SCORE
1H	1H	27	-2.1 ± 1.2
1H	1L	9	-3.8 ± 2.9
1L	1H	9	3.6 ± 1.6
1L	1L	3	6.6 ± 2.3
19H	19H	14	-1.5 ± 1.1
19H	19L	9	-2.4 ± 1.1
19L	19H	14	0.1 ± 2.4
19L	19L	9	1.7 ± 1.9

[a]Number of cultures assayed in triplicate.

Table 8 presents the mean MTT index scores (percent of control, the same combination of cells without GAT pulse *in vitro*) for the eight types of cultures assayed. Number of cultures varied due to the number of chickens available and their GAT response phenotype. Table 9 is the analysis of variance within Ea-B type based on the MTT index scores. Within the B^1 blood group, the Ir-GAT type of the APC was a significant source of variation. The Ir-GAT type of the APC within the B^{19} blood type was not significant. The responder cell Ir-GAT type was not significant within either blood group. Figure 10 shows the mean index scores by responder cell Ir-GAT type within the B^1 blood group. Figure 11 shows the results within the B^{19} blood group. None of the MTT means were significantly different for cultures within the B^{19} blood group, but the trend was the

Table 9. Analyses of variance of MTT index scores for antigen presenting cell (APC) and responder cell cultures mixed or matched by Ir-GAT type

WITHIN B1 BLOOD GROUP:

SOURCE	df[a]	MEAN SQUARE
APC GAT TYPE	1	202.5*
RESPONDER GAT	1	16.6
APC*RESPONDER GAT	1	100.5
ERROR	45	44.1

WITHIN B19 BLOOD GROUP:

SOURCE	df	MEAN SQUARE
APC GAT TYPE	1	68.7
RESPONDER GAT	1	52.1
APC*RESPONDER GAT	1	96.2
ERROR	43	34.7

[a]Degrees of freedom.
*$P<0.05$.

same as for the results in the B^1 blood group. There was a tendency for the Ir-GAT^L APC to present antigen better than the Ir-GAT^H APC.

Cell Surface Phenotype of Cultured Cells

Lymphocytes from the second assay group were also characterized for cell surface phenotype (CD4 and CD8) by immunofluorescent labelling and

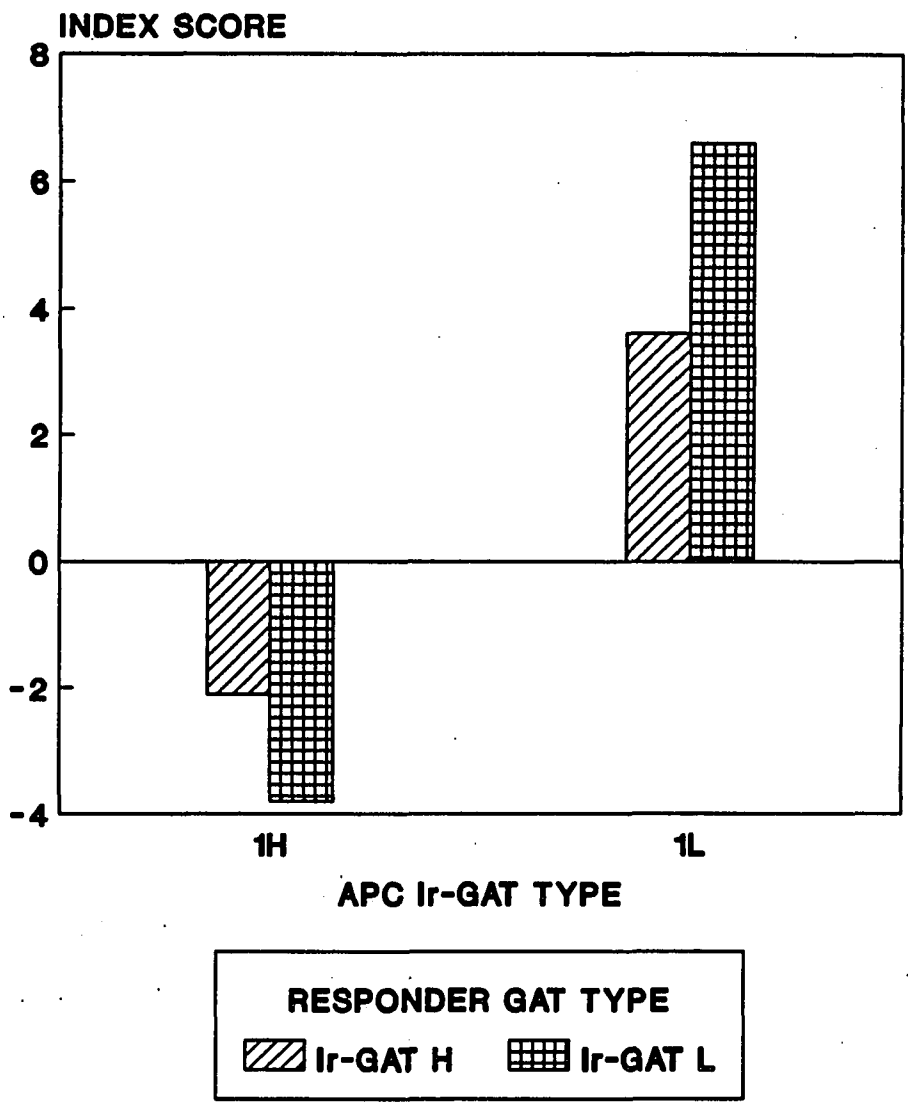


Figure 10. Mean MTT index scores within the B^1 blood group. The B^{1L} antigen-presenting cells (APC) resulted in a higher mean score than the B^{1H} APC regardless of responder cell type

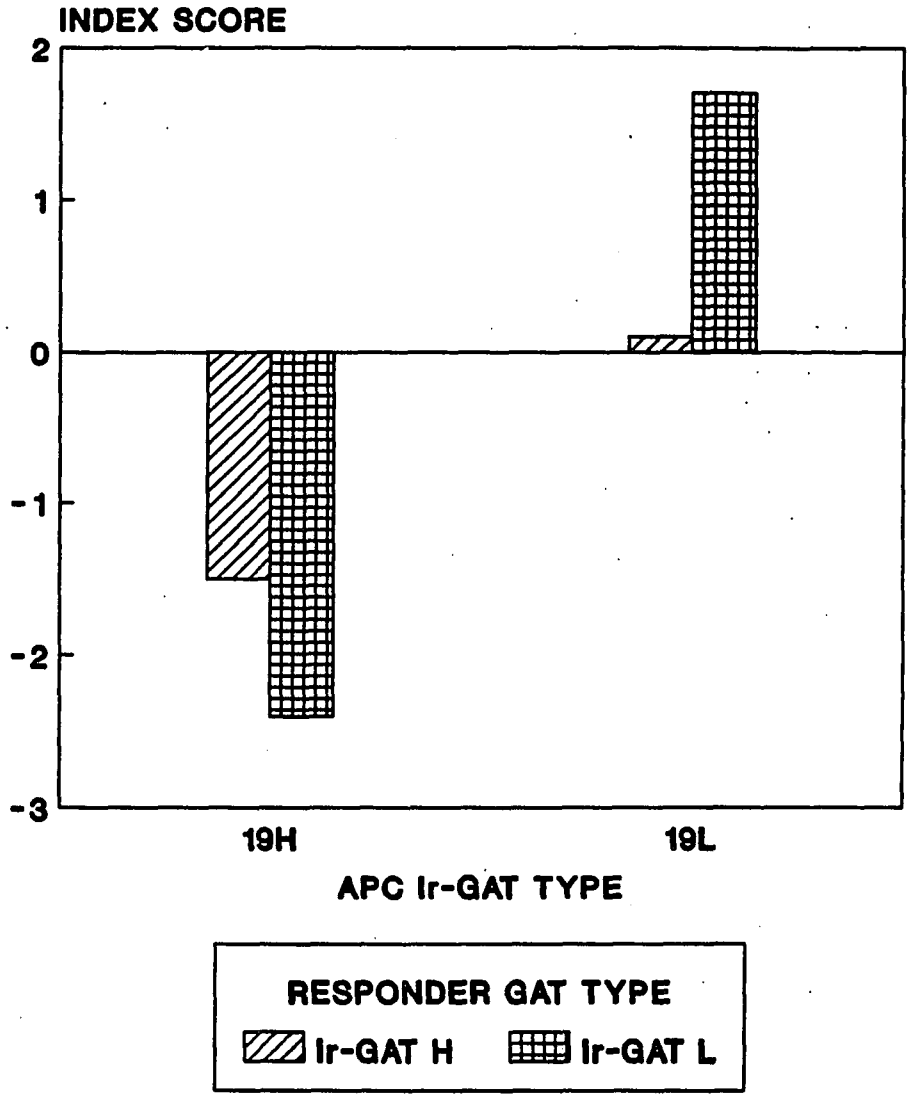


Figure 11. Mean MTT index scores within the B^{19} blood group. There was no significant difference between means based on Ir-GAT type of the antigen-presenting cells (APC) or the responder lymphocytes

flow cytometry. Cells cultured for three days at three different concentrations of GAT were labeled with monoclonal antibodies that reacted with CD4 (T helper cells) or CD8 (T suppressor/cytotoxic cells). Table 10 shows the mean percent CD4-positive cells for the S1 haplotypes at three concentrations of GAT in the medium. Figure 12 presents the data graphically. There were no differences within a haplotype by level of GAT. Within each level of GAT there were differences by haplotype (Table 11). At each GAT concentration in the medium, blood type of the donors was a significant source of variation ($P<0.02$). Sex and Ir-GAT type were not significant (Table 12).

Table 10. CD4 positive cells (percent ± standard error) for each of the four S1 haplotypes cultured at three levels of GAT. Means within a column not sharing a letter are significantly different ($P<0.05$)

	GAT CONCENTRATION IN MEDIUM					
	0 GAT		1 μg GAT		10 μg GAT	
MHC TYPE	N[a]	% $CD4^+$ LYMPHOCYTES	N[a]	% $CD4^+$ LYMPHOCYTES	N[a]	% $CD4^+$ LYMPHOCYTES
19H	8	$49.2^{A} \pm 5.3$	8	$52.6^{A} \pm 3.9$	8	$50.4^{A} \pm 4.0$
19L	8	$47.6^{A} \pm 4.1$	8	$48.9^{A} \pm 3.9$	8	$49.2^{A} \pm 3.6$
1H	8	$31.5^{B} \pm 2.8$	7	$36.0^{B} \pm 2.5$	8	$32.4^{B} \pm 2.7$
1L	8	$40.5^{AB} \pm 5.0$	8	$44.0^{AB} \pm 4.9$	8	$44.0^{A} \pm 4.6$

[a]Number of each haplotype assayed.

Table 11. Analysis of variance by concentration of GAT in the medium for percent CD4-positive cells

	0 μg GAT/WELL		1 μg GAT/WELL		10 μg GAT/WELL	
Source	df[a]	MS[b]	df	MS	df	MS
Haplotype	3	520.1*	3	381.0*	3	539.5*
Error	28	155.7	27	122.6	28	115.8

[a]Degrees of freedom.
[b]Mean square.
*P<0.05.

Table 12. Analysis of variance by GAT concentration in the medium for percent CD4-positive cells

	0 μg GAT/WELL		1 μg GAT/WELL		10 μg GAT/WELL	
SOURCE	df[a]	MS[b]	df	MS	df	MS
SEX	1	0.1117	1	21.0	1	10.1
BLOOD TYPE	1	1113.3*	1	864.8*	1	905.0*
Ir-GAT TYPE	1	101.4	1	17.6	1	223.8
ERROR	28	163.6	27	131.5	28	127.1

[a]Degrees of freedom.
[b]Mean square.
*P<0.05.

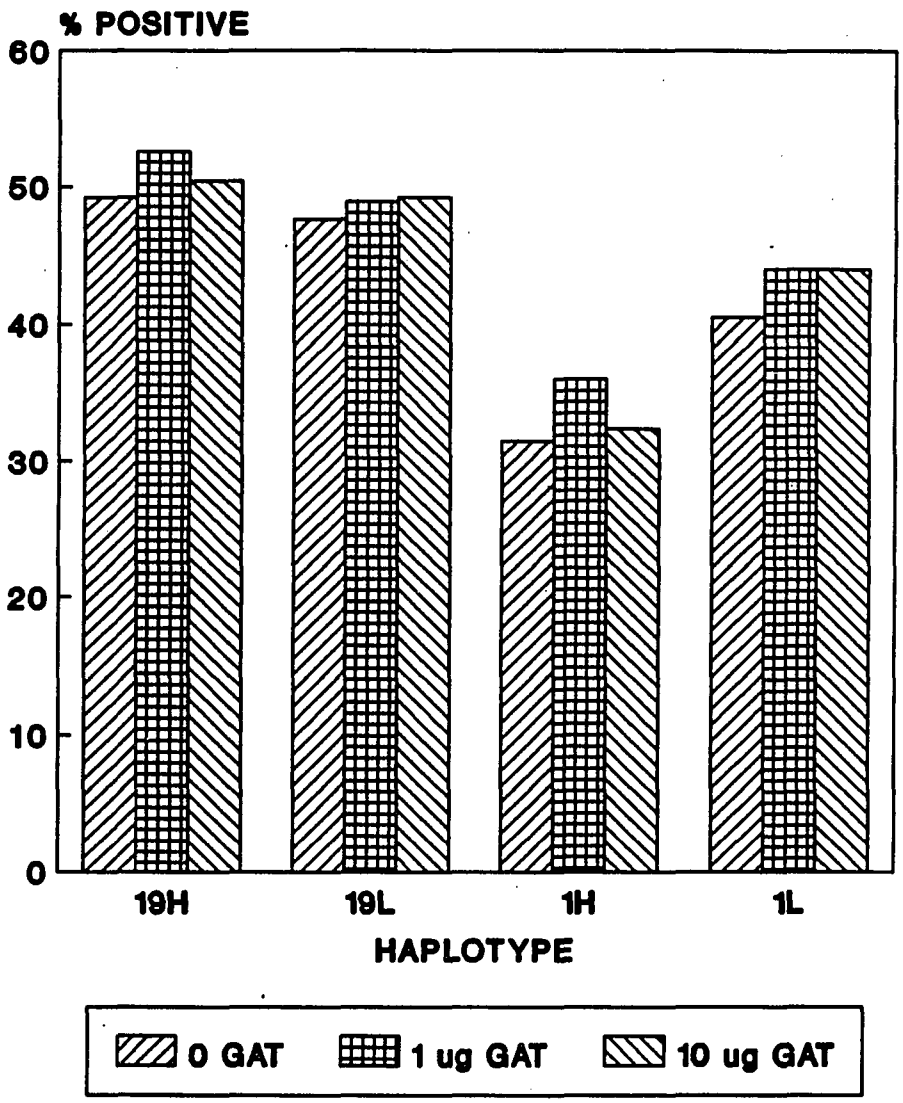


Figure 12. Mean percent CD4-positive cells by MHC haplotype of lymphocytes cultured at three different concentrations of GAT. Flow cytometry of cells labeled with anti-CD4 antibody

When results are analyzed within Ea-B type there are differences between Ir-GAT types (Table 13). Figure 13 shows the mean percent CD4-positive cells within the B^1 Ea-B type. At the 0 and 1 μg GAT levels there was no difference in CD4 levels between Ir-GAT^H and Ir-GAT^L cells. At the 10 μg GAT level there is a difference in CD4 between Ir-GAT^H and Ir-GAT^L chickens within the B^1 blood group. There were higher numbers of CD4-positive cells from B^1 Ir-GAT^L chickens.

Table 13. Analyses of variance for percent CD4-positive cells by GAT concentration within blood group

WITHIN B^1 BLOOD GROUP:

	0 μg GAT/WELL		1 μg GAT/WELL		10 μg GAT/WELL	
SOURCE	df[a]	MS[b]	df	MS	df	MS
SEX	1	5.8	1	0.7	1	84.0
Ir-GAT TYPE	1	199.3	1	194.1	1	617.8*
ERROR	13	142.5	12	131.6	13	117.4

WITHIN B^{19} BLOOD GROUP:

	0 μg GAT/WELL		1 μg GAT/WELL		10 μg GAT/WELL	
SOURCE	df	MS	df	MS	df	MS
SEX	1	2.3	1	2.6	1	1.6
Ir-GAT TYPE	1	10.4	1	54.8	1	6.0
ERROR	13	192.1	13	132.9	13	125.4

[a]Degrees of freedom.
[b]Mean square.
*$P<0.05$.

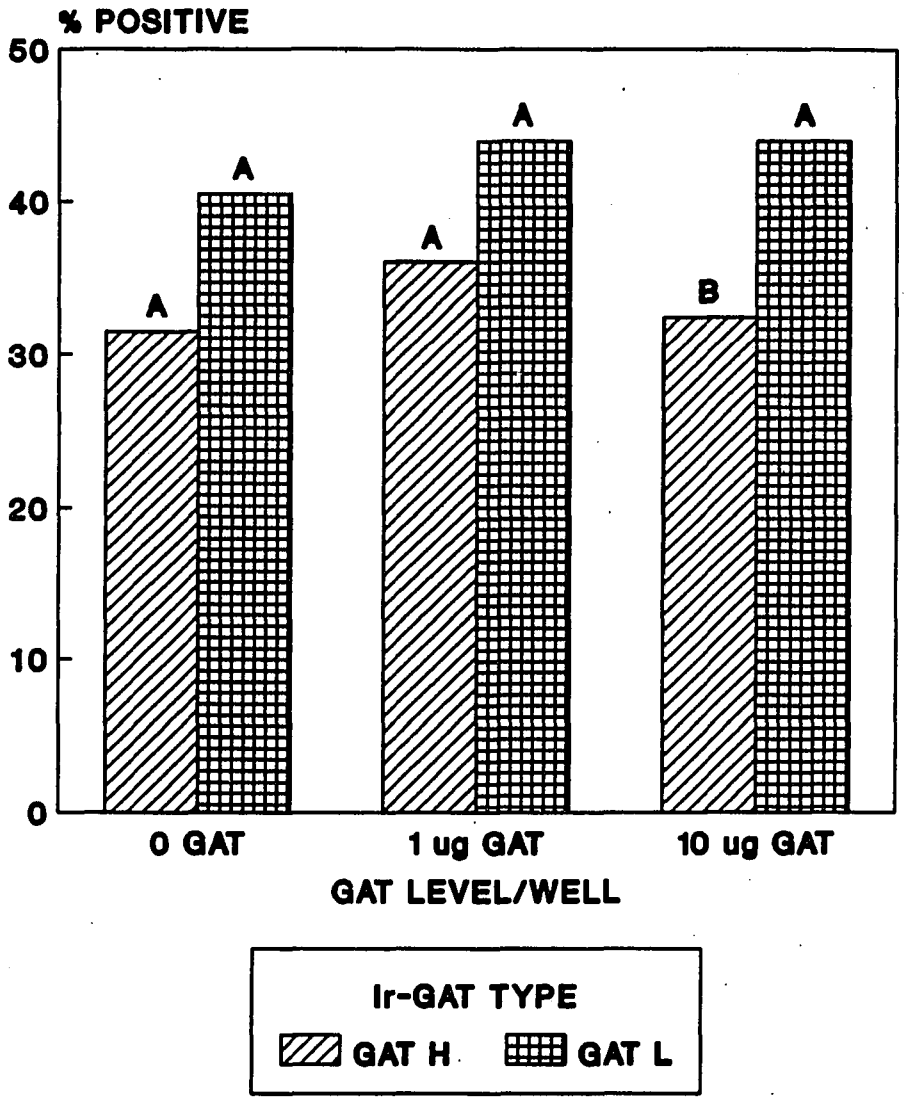


Figure 13. Mean percent CD4-positive cells within the B^1 blood type. Columns within a level of GAT not sharing a letter represent means that are significantly different ($P<0.05$)

There was no difference in percent CD4-positive cells within the B19 Ea-B type (Table 13 and Figure 14) between cells of different Ir-GAT type at any level of GAT. Figure 15 presents the mean percent of CD4-positive cells in cultures pooled by Ea-B type. At the 1 and 10 μg levels of GAT there are differences between means of the Ea-B^{19} and Ea-B^{1} groups. The lymphocytes from the B^{19} haplotype chickens had a higher percentage of CD4 positive cells compared to B^{1} chickens.

Results of labelling with an anti-CD8 monoclonal antibody are presented in Table 14 and Figure 16 as mean percent CD8-positive cells. Within a haplotype the means were not different by level of GAT. There

Table 14. CD8-positive cells (percent ± standard error) for each of the four S1 line haplotypes cultured at three levels of GAT. Means within a column not sharing a letter are significantly different ($P<0.05$)

	GAT CONCENTRATION IN MEDIUM					
	0 GAT		1 μg GAT		10 μg GAT	
MHC TYPE	N[a]	% $CD8^+$ LYMPHOCYTES	N[a]	% $CD8^+$ LYMPHOCYTES	N[a]	% $CD8^+$ LYMPHOCYTES
19H	16	17.7^{B} ± 1.3	16	17.1^{B} ± 1.2	16	16.5^{B} ± 1.5
19L	16	22.7^{A} ± 1.9	16	22.1^{A} ± 1.5	16	22.8^{A} ± 1.9
1H	16	15.8^{B} ± 1.0	15	14.8^{B} ± 1.2	16	15.7^{B} ± 1.0
1L	16	15.4^{B} ± 1.0	16	16.2^{B} ± 0.9	16	15.6^{B} ± 0.9

[a]Number of each haplotype assayed.

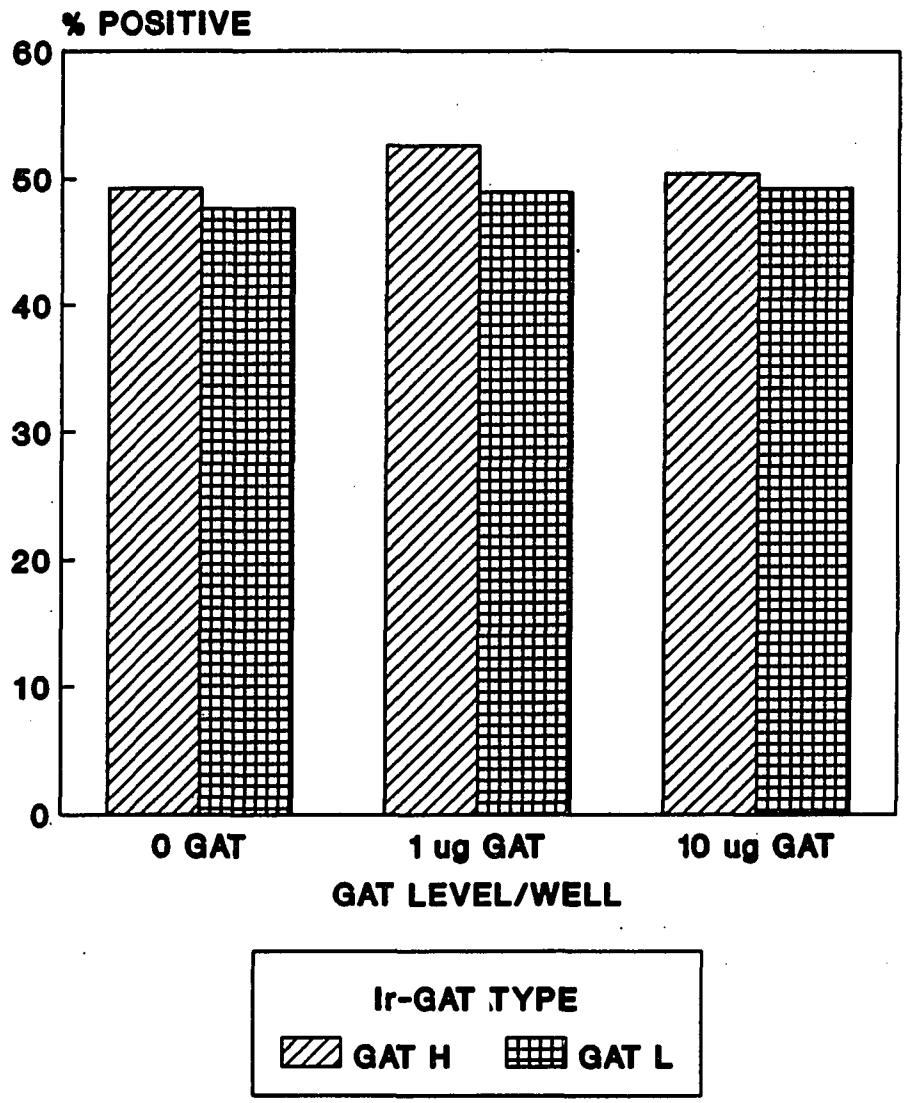


Figure 14. Mean percent CD4-positive cells within the B^{19} blood group

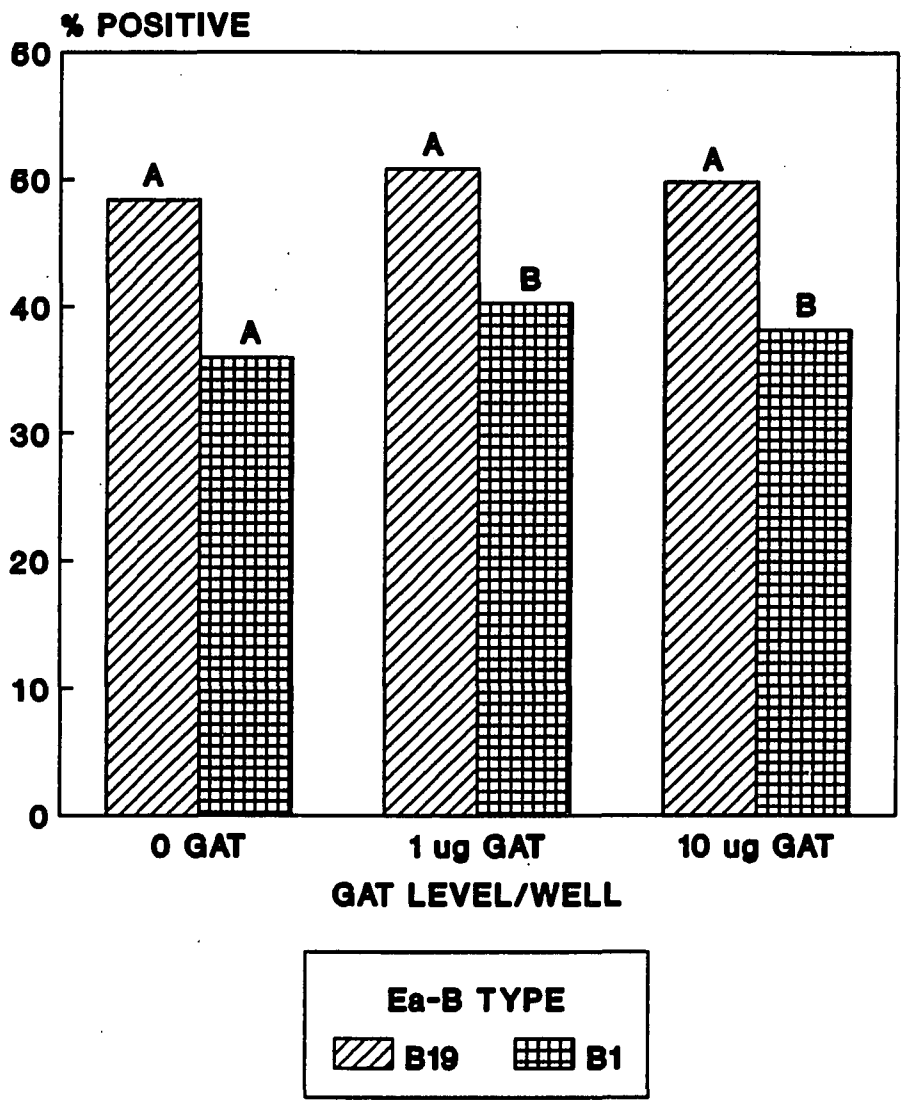


Figure 15. Mean percent CD4-positive cells, results pooled within blood type. Columns within a level of GAT not sharing a letter represent means that are significantly different ($P<0.05$)

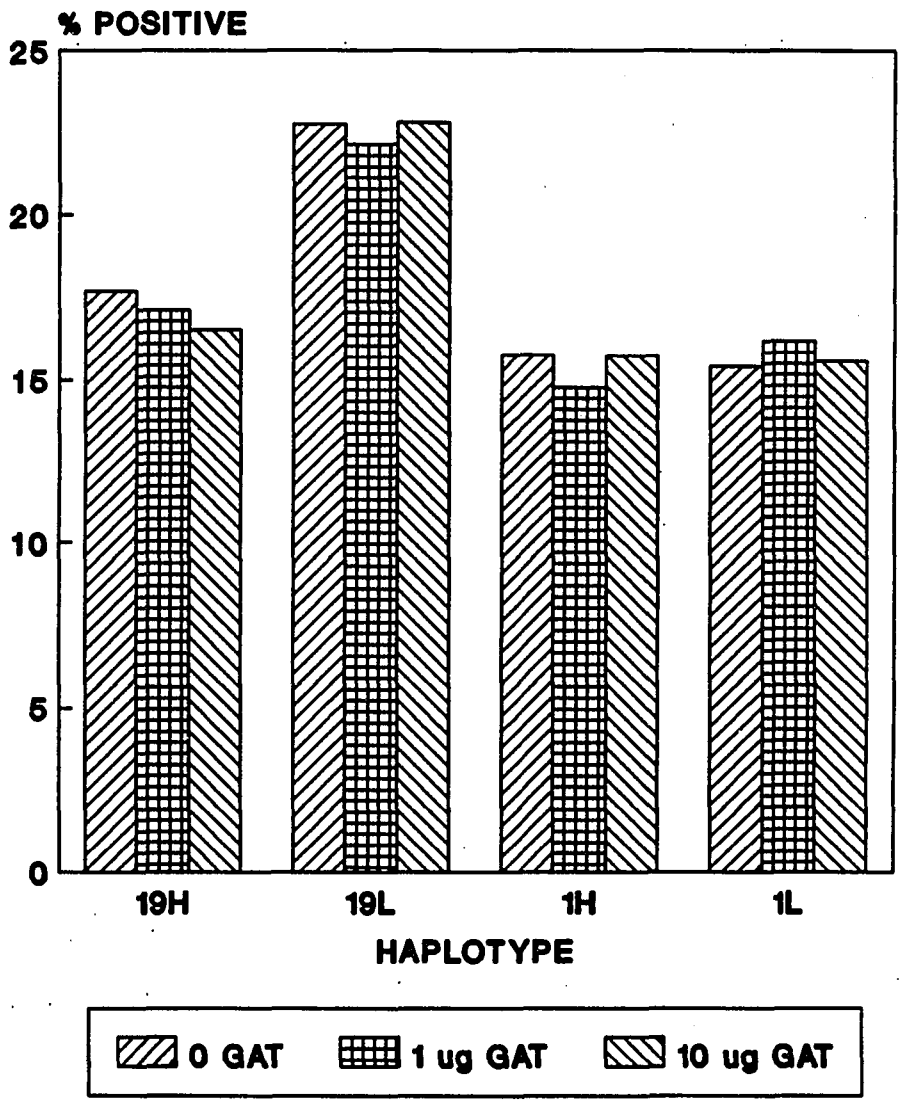


Figure 16. Mean percent CD8-positive cells by MHC haplotype of lymphocytes cultured at three different levels of GAT. Flow cytometry of cells labeled with anti-CD8 antibody

were differences between haplotypes within a level of GAT (Table 15). The cells from B^{19L} chickens had a higher percentage of CD8 positive cells at each level of GAT (Table 16). At the 10 μg GAT level, Ir-GAT type of the cells was also significant.

Table 15. Analysis of variance for percent CD8-positive cells for each concentration of GAT in the medium

	0 μg GAT/WELL		1 μg GAT/WELL		10 μg GAT/WELL	
Source	df[a]	MS[b]	df	MS	df	MS
Haplotype	3	183.0*	3	139.0*	3	190.3*
Error	60	30.0	60	23.4	60	29.7

[a]Degrees of freedom.
[b]Mean square.
*P<0.001.

Table 16. Analysis of variance by GAT concentration for percent CD8-positive cells

	0 μg GAT/WELL		1 μg GAT/WELL		10 μg GAT/WELL	
SOURCE	df[a]	MS[b]	df	MS	df	MS
SEX	1	64.6	1	67.6	1	94.5
BLOOD TYPE	1	397.2**	1	265.1**	1	317.6*
Ir-GAT TYPE	1	82.9	1	115.3*	1	140.4*
ERROR	60	30.8	59	23.6	60	30.9

[a]Degrees of freedom.
[b]Mean square.
*P<0.05.
**P<0.001.

Results were separated by Ea-B type and analyzed. Figure 17 and Table 17 shows the results within the B^1 blood group and there were no differences associated with Ir-GAT type of the cells or the level of GAT. Figure 18 and Table 17 show the results within the B^{19} blood group. At each concentration of GAT, lymphocytes from Ir-GAT^L chickens had higher numbers of CD8-positive cells. Data pooled by Ea-B type (Figure 19) indicated that the B^{19} cultures had higher levels of CD8-positive cells than the B^1 cultures, although there were no differences due to GAT level.

Table 17. Analyses of variance for percent CD8-positive cells by GAT level and within blood group

WITHIN B^1 BLOOD GROUP:

	0 μg GAT/WELL		1 μg GAT/WELL		10 μ GAT/WELL	
SOURCE	df[a]	MS[b]	df	MS	df	MS
SEX	1	1.1	1	12.6	1	7.9
Ir-GAT TYPE	1	1.7	1	1.3	1	0.5
ERROR	29	29	28	17.1	29	14.1

WITHIN B^{19} BLOOD GROUP:

	0 μg GAT/WELL		1 μg GAT/WELL		10 μg GAT/WELL	
SOURCE	df	MS	df	MS	df	MS
SEX	1	106.6	1	70.2	1	123.6
Ir-GAT TYPE	1	202.0*	1	199.0*	1	313.1*
ERROR	29	40.9	29	28.2	29	42.9

[a]Degrees of freedom.
[b]Mean square.
*P<0.05.

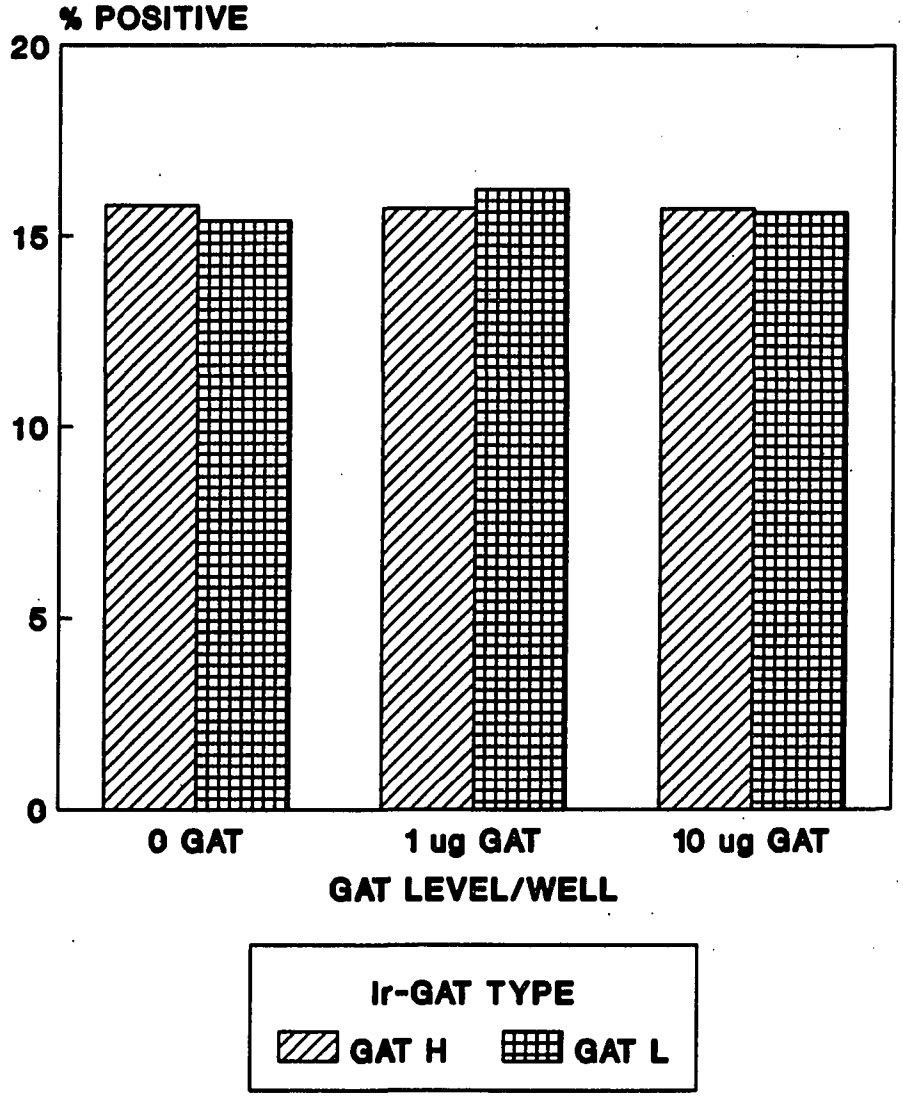


Figure 17. Mean percent of CD8-positive cells within the B^1 blood group

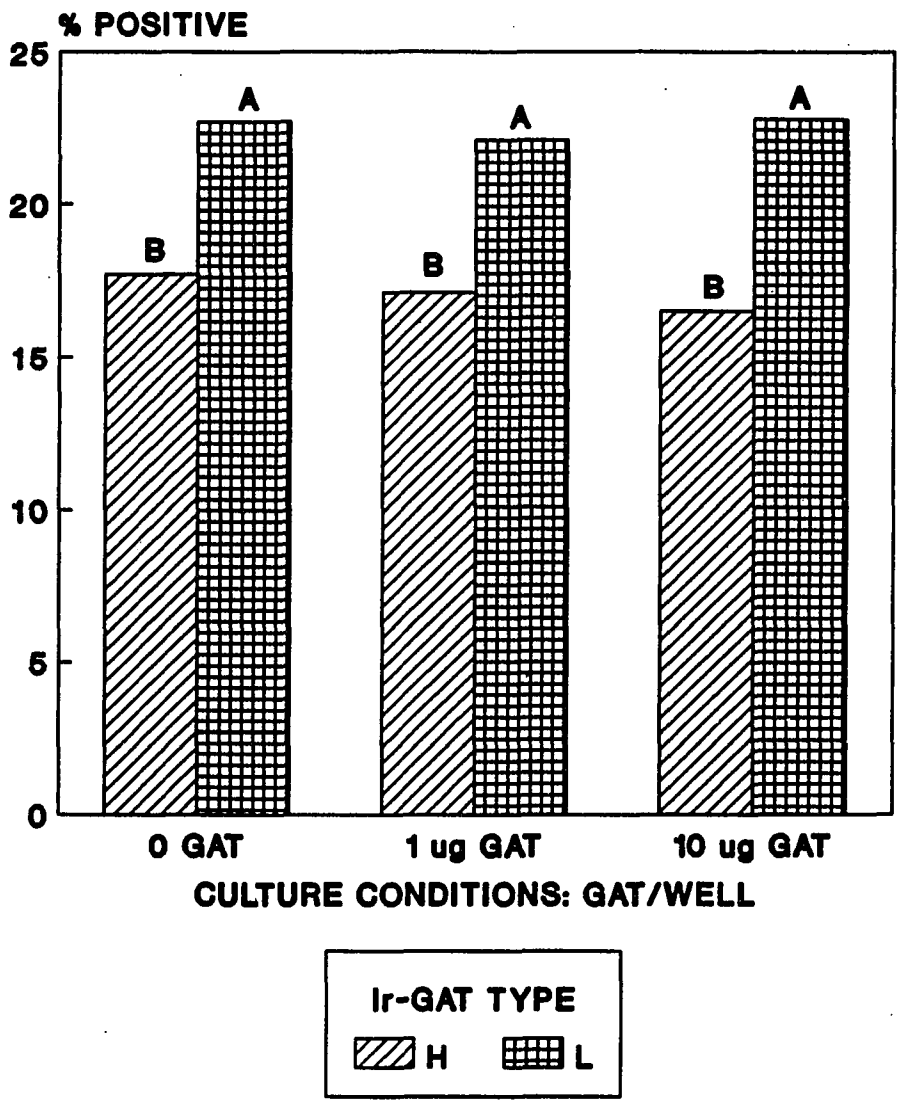


Figure 18. Mean percent of CD8-positive cells within the B^{19} blood type. Columns within a level of GAT not sharing a letter represent means that are significantly different ($P<0.05$)

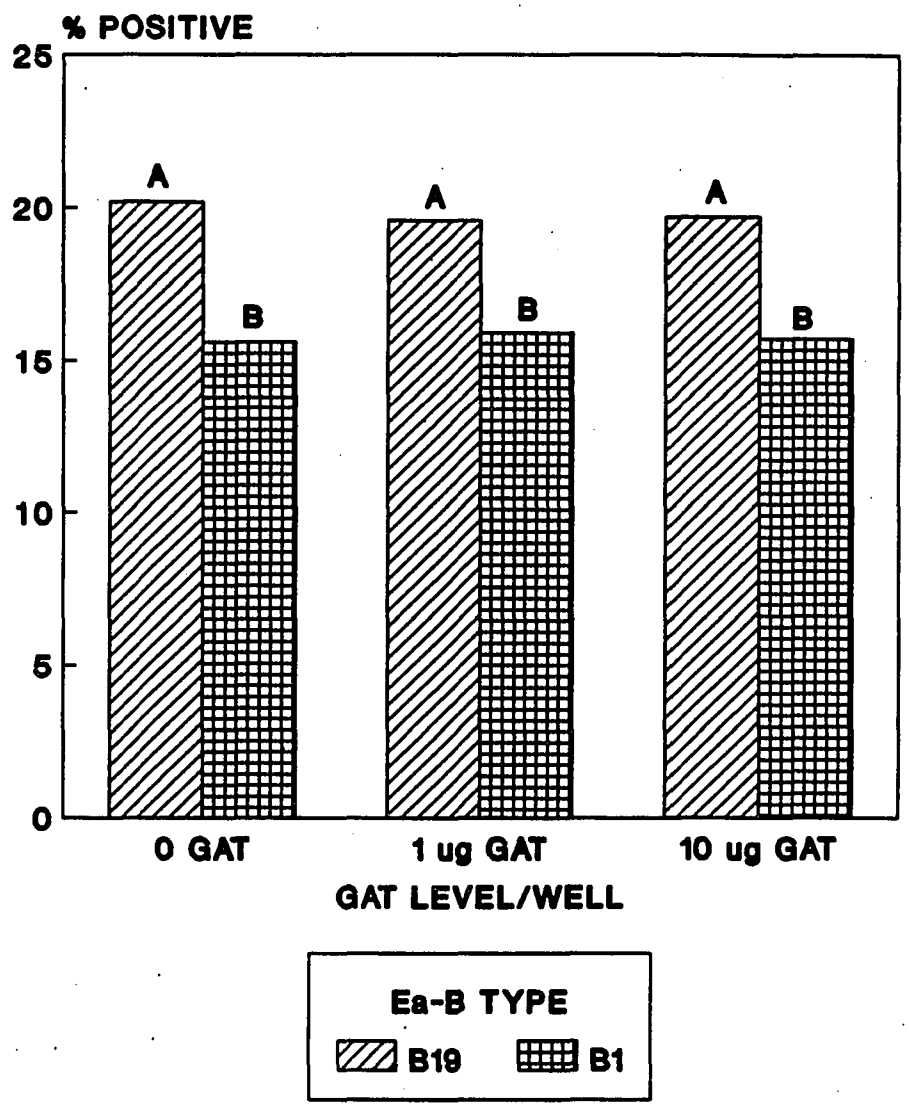


Figure 19. Mean percent of CD8-positive cells pooled by blood type. Columns within a level of GAT not sharing a letter represent means that are significantly different ($P<0.05$)

Anti-GAT Response in Progeny From Complementation Matings

Two types of F_1 crosses were made to produce progeny that could be assayed by GAT challenge for evidence of gene complementation. The first type was a within-line cross between B^{19L} and B^{1L} chickens of the S1 line and the second type was between S1 line B^{19L} chickens and GHs line B^{13} chickens. Table 18 presents the means of secondary GAT radioimmunoassay (RIA) values for chickens of MHC haplotypes within the lines. Table 19 presents the mean percent GAT bound by the F_1 progeny of the within S1 line cross. The progeny did not differ in GAT response by haplotype of sire. The mean percent bound GAT of the F_1 progeny was not different from the means of the parental sublines.

Table 18. Mean percent GAT bound (± standard error) in radioimmunoassay by secondary immune sera from the parental line types. Means not sharing a letter are significantly different ($P<0.05$)

LINE AND HAPLOTYPE	N[a]	MEAN GAT-RIA
S1 -19H	23	80.3^{A} ± 3.4
S1 -19L	25	27.4^{C} ± 5.6
S1 -1H	19	88.7^{A} ± 3.3
S1 -1L	23	35.2^{BC} ± 6.2
GHs -6	20	43.0^{B} ± 3.2
GHs -13	18	5.3^{D} ± 1.1

[a]Number assayed.

Table 19. Mean percent GAT bound (± standard error) in radioimmunoassay by secondary immune sera from the F_1 progeny of crosses within the S1 line

TYPE OF F_1 MATING	N[a]	MEAN GAT-RIA
S1-1L SIRE X S1-19L DAM	40	27.1 ± 4.4
S1-19L SIRE X S1-1L DAM	69	18.4 ± 2.7

[a]Number of progeny tested.

Table 20 presents the percent GAT bound means for the F_1 progeny of the between line crosses. GAT response means were not significantly different by sire MHC haplotype. The progeny mean was significantly higher ($P<0.05$) than the mean percent GAT bound by the parental sublines.

Table 20. Mean percent GAT bound (± standard error) in radioimmunoassay by secondary immune sera from the F_1 progeny of between line crosses

TYPE OF F_1 MATING	N[a]	MEAN GAT-RIA
S1-19L SIRE X GHs-13 DAM	57	48.2 ± 3.6
GHs-13 SIRE X S1-19L DAM	82	53.2 ± 3.4

[a]Number of progeny tested.

Progeny from the between-line crosses were backcrossed to the parental sublines (GHs-B^{13} and S1-B^{19L}), as well as mated *inter se*. The resulting progeny were of three different blood types (B^{19} homozygotes, B^{13} homozygotes, and B^{19}/B^{13} heterozygotes). The F_2 progeny were

assayed for secondary GAT response. Table 21 and figure 20 show the mean percent GAT bound for the three different groups. No matter from what type of mating they derived, the B^{13} homozygotes produced low levels of antibody when challenged with GAT.

Table 21. Mean percent GAT bound (± standard error) in radioimmunassay by secondary immune sera from the F_2 progeny by blood type. Means not sharing a letter are significantly different ($P<0.05$)

BLOOD TYPE OF F_2 PROGENY	N[a]	MEAN GAT-RIA
B^{19}/B^{19}	95	$66.6^{A} \pm 3.2$
B^{19}/B^{13}	195	$71.9^{A} \pm 2.5$
B^{13}/B^{13}	96	$4.6^{B} \pm 1.4$

[a]Number assayed.

Table 22 shows the analysis of variance for GAT response by mating type (F_1 backcrossed to the S1-19L roosters or hens, F_1 backcrossed to GHs-13 roosters or hens, and F_1 crossed with F_1). Sire was a significant source of variation for GAT response of progeny from S1-19L backcrosses, but wasn't significant for the other two mating types. GAT response of progeny produced from F_1 X F_1 and F_1 X GHs-13 matings differed by their Ea-B (blood) type. Homozygous B^{13} progeny were significantly lower in GAT response than homozygous B^{19} or heterozygous B^{19}/B^{13}. Sires used in backcrosses to the S1 or GHs lines were of two blood types (B^{19}/B^{13} from the F_1 generation and B^{19}/B^{19} or B^{13}/B^{13} from the parental lines). Table 23 shows the analysis of variance by mating type including sire Ea-B as a

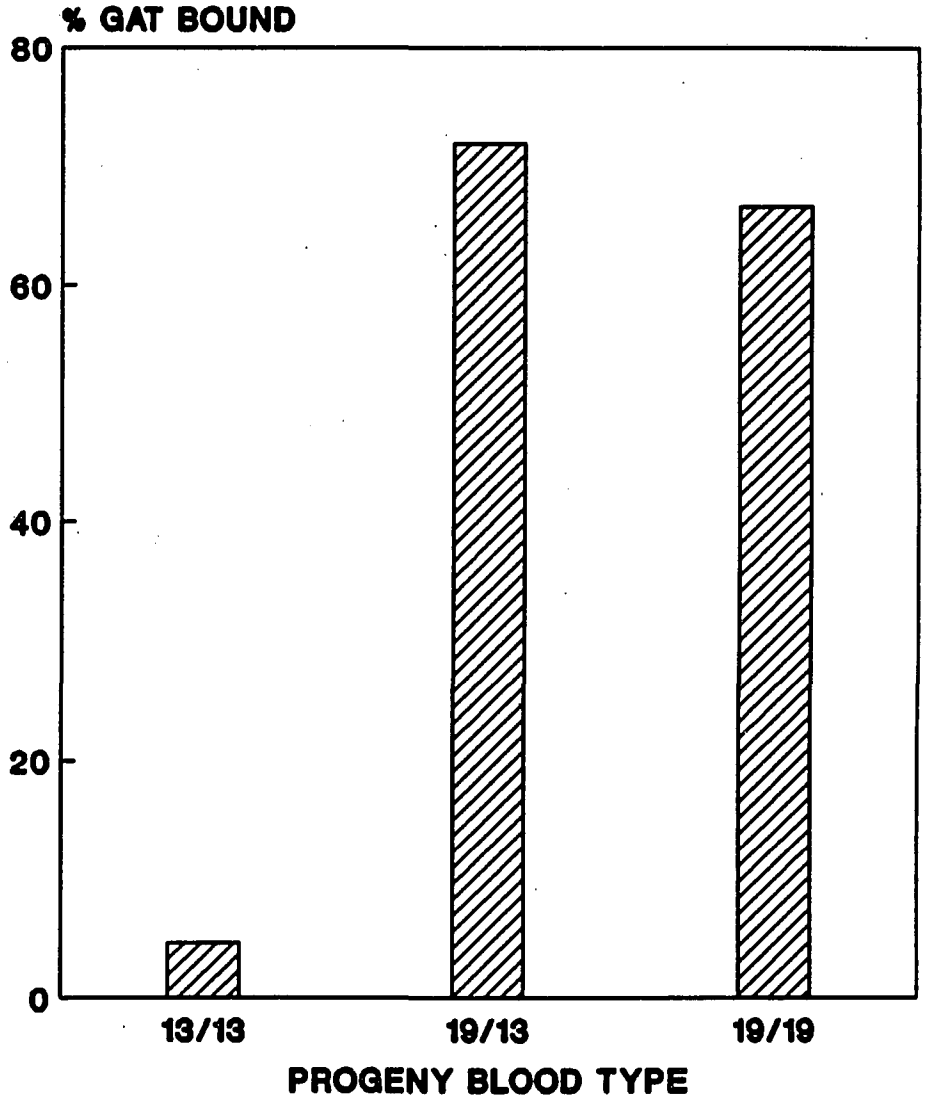


Figure 20. Mean secondary GAT antibody response (percent bound) measured by radioimmunoassay for the F_2 progeny from backcross and *inter se* matings

Table 22. Analysis of variance for the percent GAT bound by F_2 progeny produced by backcrosses or *inter se* matings

	BACKCROSS TO 19L		BACKCROSS TO 13		F_1 CROSSED TO F_1	
SOURCE	df[a]	MS[b]	df	MS	df	MS
SIRE	9	2972*	10	127	7	405
PROGENY Ea-B	1	2133	1	207707**	2	75529**
DAM(SIRE)	4	278	8	248	5	955
ERROR[c]	118	990	105	269	113	568

[a]Degrees of freedom.
[b]Mean square.
[c]Error for testing DAM(SIRE) was SIRE mean square.
*P<0.01.
**P<0.0001.

Table 23. Analysis of variance for percent GAT bound by F_2 progeny

	BACKCROSS TO 19L		BACKCROSS TO 13	
SOURCE	df[a]	MS[b]	df	MS
SIRE Ea-B TYPE	1	14291**	1	88
PROGENY Ea-B TYPE	1	4452*	1	234679***
ERROR	130	1009	122	258

[a]Degrees of freedom.
[b]Mean square.
*P<0.05.
**P<0.001.
***P<0.0001.

as a source of variation. The sire Ea-B type resulted in significant differences in GAT response of their progeny. The progeny of B^{19}/B^{19} sires had higher GAT response than the progeny of B^{19}/B^{13} sires and the heterozygous progeny (B^{19}/B^{13}) were lower than the homozygous (B^{19}/B^{19}) progeny.

DISCUSSION

Value of Ir-GAT as a Marker for Disease Resistance

The importance of Ir-GAT as a marker for disease resistance has been well-investigated in the chicken. An example in the appendix of this dissertation presents the results of challenging S1 chickens with Marek's disease virus (MDV). The association between high response to GAT and resistance to Marek's disease remained through two generations of chickens tested. This association was confirmed when an F_2 population of chickens was challenged with MDV. Similar disease resistance has been seen when chickens unrelated to the S1 line have been selected for humoral immune response to GAT. Pevzner *et al.* (1989a, 1989b) conducted a divergent selection experiment within two types of commercial chickens (meat and egg-type). After developing sublines that were low or high responders to GAT, the chickens were challenged with MDV, Rous sarcoma virus, or *Staphylococcus aureus*. The Ir-GAT high lines were more resistant to the effects of the pathogens than the low responder sublines. Equally important, selection for Ir-GAT did not result in any significant change in performance characteristics of economic value. Pevzner attributed response to GAT to a major immune response gene, but did not report on any changes in frequency of MHC haplotypes in lines under selection.

The benefits of Ir-GAT^H coupled with the MHC effects do impart some advantage to the survival of the chicken. Selection for disease-resistant MHC types is complemented by selection for high response to GAT. For example, incidence of Marek's disease in MDV-challenged chickens is low in

B^1 haplotype S1 chickens and is lower still in B^1 chickens that are high responders to GAT. This effect is also seen when more susceptible chickens (S1-B^{19}) are challenged with MDV; that is, the Ir-GAT^H within the blood type are more resistant to incidence of Marek's disease (Steadham *et al.* 1987). Although the results of selecting for GAT response are clear, the immunological mechanisms involved have not been investigated in the chicken and are poorly defined. This dissertation research addressed the mechanisms involved in the observed differences in specific antibody titers after chickens are challenged with GAT.

B-L Genes As Ir-GAT Antigens

In mammalian systems, differences in immune response can often be attributed to immune response (Ir) genes that are within the MHC (McDevitt *et al.* 1972). When Pevzner *et al.* (1978) first reported the recombination between Ir-GAT and Ea-B (B-F/B-G), a possible explanation was that the recombination was within the MHC, using the hypothesis that Ir-GAT is controlled by a specific Ir gene in the MHC of the chicken.

Attempts to locate the recombination within the MHC have never supported this hypothesis. Extensive attempts to raise alloantisera (reported in this dissertation) were unsuccessful. This could be due to the lack of recombination within the MHC of the S1 sublines but could also be due to the difficulty of producing antibody to B-L antigens. The latter is not thought to be the case, because of the rigor of the testing applied. Literally hundreds of chickens were immunized in attempts to produce B-L alloantisera. The methods used to screen the antisera became

increasingly more sophisticated and sensitive. These methods were able to detect B-G and B-F antigen differences, but never resulted in evidence of B-L antibody in the serum that was assayed, and thus no serological evidence could be provided to support the hypothesis that Ir-GAT genes encode MHC class II gene products.

Recent developments in molecular genetics have enabled the direct investigation of the DNA for evidence of recombination. Pitkovski *et al.* (1989) used a chicken class II probe to examine the DNA of S1 chickens for evidence of restriction fragment length polymorphism (RFLP) that could be associated with Ir-GAT differences. Although 15 different endonucleases were used to generate fragments, none of the resulting RFLP could be associated with Ir-GAT. Similar results have been seen when a class I probe is used to examine DNA from S1 chickens (Chen and Lamont 1991). The RFLP generated are associated with Ea-B type and not Ir-GAT type.

Pitkovski *et al.* (1989) reported that there were three possible explanations that could account for the lack of RFLP associated with GAT response. First, the choice of endonucleases could have excluded the polymorphic locus. There are available commercially several hundred endonucleases with different restriction sites. It would not only be extremely expensive to try them all, it would consume a great deal of time. Large stretches of DNA can be examined with a single enzyme and probe. The probability that a recombination would be missed when testing against 15 different endonucleases becomes exceedingly small. However, there have been instances in mice were a single nucleotide substitution has resulted in an amino acid substitution that resulted in a difference

in immune response phenotype (Brown *et al.* 1986).

The second possibility Pitkovski *et al.* (1989) proposed was that immune response to GAT was mediated by the class II α gene. While it is possible that B-L α may mediate immune response to GAT, it would be likely that the class II β probe would still result in RFLP if the class II α and β genes are in close proximity to each other in chickens as they are in mice (Kaufman *et al.* 1984). At present, there are no chicken B-L α probes available and there is not enough homology between mammalian class II α probes and chicken DNA to be able to use the mammalian probes (Guillemot *et al.* 1988).

The third possibility proposed by Pitkovski *et al.* (1989) was that control of immune response to GAT was outside the MHC. The boundaries of the chicken MHC are not firmly established. While the nucleolar organizer region (NOR) marks one end of the MHC, the other end has not been defined (Guillemot *et al.* 1988). It could still be possible that Ir-GAT is within or linked to the end of the chicken MHC that has not been identified.

The classical explanation of immune response mediation through Ir genes within the MHC may not be applicable in the chicken. There are genes that are within the chicken MHC that are not like the traditional class I or class II genes seen in mammals or chickens. One of these genes (identified by probe C12.3) has homology to the β subunit of the human guanine nucleotide-binding protein (G protein) which is not found within the mammalian MHC (Guillemot *et al.* 1989a). The G proteins function to transmit signals from cell surface receptors through the cell membrane and have been associated with transmembrane signalling in immune cells (Neer

and Clapham 1988, Harnett and Klaus 1988). Monocytes, neutrophils, T lymphocytes, and B lymphocytes undergo receptor mediated activation steps that involve the G protein (Harnett and Claus 1988). It is not known if alleles exist for the β subunit gene although Guillemot *et al.* (1989a) speculated that alleles may exist and could explain differences in resistance to Marek's disease associated with some MHC haplotypes (it could also explain differences seen in mitogen activation). There are at least six other non-class I/class II genes found within the chicken MHC that have not been characterized yet (Guillemot *et al.* 1988). Some of these unidentified genes are proposed to have an effect on immune reactivity.

GAT-induced *In Vitro* Proliferation of Antigen-primed Lymphocytes

Measurements of *in vitro* reactivity of antigen (GAT)-primed T lymphocytes were undertaken to determine if there were differences in proliferation that could explain the differences in humoral GAT response. T cells play important accessory roles in the immune response leading to antibody production (see Figure 1, page 18). Three different levels of GAT were used in the medium to stimulate lymphocytes from GAT primed chickens (0, 1 μg, and 10 μg GAT per well). Proliferation (as measured by MTT assay) did not differ within S1 line haplotypes by level of GAT. The conclusions drawn from the results of assaying proliferation *in vitro* indicate that T cell reactivity to GAT is not likely to be the cause of different antibody response in the whole animal. This is in contrast to what is seen when similar experiments are conducted with mouse cells

(Kimoto and Fathman 1980, Gougeon and Theze 1983).

Kimoto and Fathman (1980) used responder and non-responder lines of mice as sources of GAT-primed lymphocytes. Subsequent secondary stimulation *in vitro* resulted in proliferation of responder mouse cells above the levels seen for cells in wells without GAT. The effect could be seen with doses of GAT as low as 2 μg/well and increased up to levels of 40 μg GAT/well. Cultures of chicken cells stimulated with 20 μg GAT/well died before proliferation could be measured (at three days).

The differences seen in cell proliferation were not associated with the level of GAT in the medium. The amount of proliferation was the same for wells with antigen as wells without antigen. A set of cultures were incubated for five days (duplicate cultures were assayed after the usual three days) to allow more time for proliferation to occur. Results were inconclusive at the longer incubation time as the cells from most chickens died before the end of incubation and others were greatly decreased. The range of responses among chickens of the same MHC haplotypes were very wide. Individual chickens were found that proliferated well *in vitro* regardless of their haplotype. There was no association with the actual GAT antibody response (as measured by radioimmunoassay) at the whole animal level and the ability of the isolated cells to proliferate *in vitro*.

The limited diversity of antigenic sites on the GAT molecule, although useful for determining immune response differences in the whole animal, were likely not sufficient for stimulating cells *in vitro*. The population of cells assayed *in vitro* is not the complete population of

cells available *in vivo*. Similar cultures of chicken cells using a different antigen have resulted in proliferation above control levels (Vainio *et al*. 1988). Vainio *et al*. used keyhole limpet hemocyanin (KLH) as an antigen and cells receiving secondary exposure to the antigen *in vitro* proliferated. KLH presents a more diverse set of antigenic determinants than GAT and would likely result in a broader population of T cells that could be stimulated by the antigen. The number of lymphocytes responding to the antigen may not be as important, however, as what functional type of lymphocytes are responding to the antigen. If the mechanisms of immune suppression were induced (as mediated by CD8-positive T cells), there would not necessarily be evidence of proliferation, or a proliferation of CD8-positive cells could result in suppressed (low) antibody response at the whole animal level. On the other hand, the helper T cell populations (bearing the CD4 phenotypic marker) that result in augmented immune response could also exert their effects without noticeable increases in cell numbers.

The assay results might have been different if exogenous interleukin 2 (IL-2) had been added to the culture medium. The IL-2 would have allowed longer incubation times for proliferation differences to occur and there is a synergistic effect when antigen and IL-2 are used to induce proliferation. However, the drawbacks of including IL-2 in the medium prevented its use in this system. At present, there is no consistent source of chicken IL-2 other than using conditioned medium (the medium that Con-A stimulated T cells have secreted IL-2 into) and mammalian IL-2 is not biologically active on chicken lymphocytes (Schauenstein *et al*.

1982). Conditioned medium not only contains IL-2 but also any Con-A that can not be removed or inactivated and all other soluble factors secreted after mitogen stimulation. When conditioned medium is used as a growth supplement, there is the possibility that the Con-A becomes more important in inducing proliferation than the antigen that is in the medium. If the Con-A is inactivated totally, there is still the possibility that the proliferation that is induced primarily reflects the response of the cultured cells to IL-2 (which can be accounted for with the proper controls). An additional problem is that the biological half-life of IL-2 activity is short and therefore can vary widely in batches of conditioned medium.

Antigen-Presenting Cell Assay

There are examples in which differences in levels of antibody produced can be attributed to differences in the ability of antigen presenting cells (APC) to process and present antigen. Some lines of "Biozzi" mice produce low levels of antibody based on the inability of their APC to present antigen to responsive T cells (Adorini and Doria 1983). The macrophages (APC) from low responder mice processed antigen so rapidly that when the processed antigen was expressed on the cell surface it was in a form that would not cause T cells to respond. The T cells from low responder mice could be induced to proliferate *in vitro* when the APC were exposed to large quantities of the antigen. The APC were thought to express a less processed antigen fragment on their surface that the T cells could recognize. The APC with faster catabolic rates (from low

responder mice) resulted in low antibody production in the whole animal.

When lymphocytes from GAT-primed chickens were cultured with GAT pulsed APC from Ir-GAT high or low chickens, the APC from the Ir-GAT^{L} chickens were better able to stimulate the responder cells regardless of responder cell Ir-GAT type. If an antigen-presenting cell defect were responsible for the differences seen in humoral immune response to GAT, the expectation would be for the APC from Ir-GAT^{H} to present antigen better than the APC from Ir-GAT^{L}. While the deviation from expected results could have been due to chance or reactivity of cells toward other alloantigen systems on the surface of the cells, the same effects should have randomly influenced the responder T cells of either Ir-GAT type. For example, if the proliferation were due to reactivity toward alloantigens there would be no reason to think that the alloantigens existed in one subline and not the others.

Immune reactions can be broadly divided into those that are primarily humoral (mediated by antibody) and reactions that are primarily cellular (mediated through the actions of activated cytotoxic cells). The *in vitro* APC assay is an immune reaction that is mediated by the cells involved and as a result may not be a reflection of the mechanisms of *in vivo* antibody production. The requirements for antigen presentation for humoral immune reactions (antibody production) *in vivo* are different than the requirements for stimulating T cells *in vitro*. Therefore, it is not completely unexpected that APC from Ir-GAT^{H} birds cause lower proliferation of responder cells *in vitro* as the mechanisms involved are inversely related. Estimates of quantitative genetic parameters in

chickens selected for immune response traits have shown that the cell mediated and humoral mediated traits have negative or low phenotypic correlations (Cheng and Lamont 1991, Cheng *et al.* 1991). Selection for high antibody response would have a tendency to lower the immune reactions mediated by cellular mechanisms.

Defective antigen presentation is not always the cause of low antibody production. Of the five different lines of "Biozzi" mice (selected for divergent antibody production to different antigens) tested, deficient antigen presentation was a likely cause of low antibody production in only two of the lines (Biozzi *et al.* 1984, Mouton *et al.* 1984). Thomas and Hoffman (1982) used responder and non-responder guinea pigs to angiotensin to examine the role of APC in immune response. Their results indicated there was no evidence of defective antigen presentation. Their results indicated that response was likely regulated by the T cells involved. Clearly, choice of antigen system and experimental animal can result in different results. Results obtained from experiments conducted *in vitro* may not be directly comparable with the events that occur *in vivo*, but the advantages of separating the component cells of the immune reaction make such assays desirable. The reactions between immune cells are obscured in the whole animal and only the end results (such as antibody produced or health of the animal) can be evaluated. In the case of chicken response to GAT, the APC assayed *in vitro* do not account for the differences in antibody levels seen in the whole animal.

Phenotypic Markers on Cultured Cells

Lack of proliferation in response to secondary antigen exposure *in vitro* may not be as significant an indicator of antibody producing potential as the functional type of the cells that proliferate in response to the antigen. For example, cell numbers would not have to increase significantly if all of the cells produced were antigen specific and of suppressor or helper functional type (bearing the CD8 or CD4 surface marker respectively). Gougeon and Theze (1983) were able to cause *in vitro* T cell proliferation in response to GAT in non-responder mice by first deleting the cell cultures of suppressor T cells. Pierce *et al.* (1988) showed that T-cell populations that were induced in GAT non-responder mice were specific for the antigen and had the phenotypic marker (CD8) of suppressor cells. Non-response to GAT in mice is apparently mediated through antigen specific suppressor T cells. The results of these two experiments also provide evidence against the possibility of deletions in T cell populations that recognize specific antigen. In both cases, T cells from non-responder mice could respond to the antigen under the proper conditions.

The percentages of CD4- or CD8-positive S1 chicken cells did not change with different levels of GAT in the culture medium. There were difference in percentages by MHC haplotype. The cells from B^{1H} chickens had the lowest number of CD4-positive cells at each level of GAT and the B^{19L} haplotype had the highest number of CD8-positive cells. The cells from B^{19} chickens had higher levels of CD4- and CD8-positive cells compared to cells from B^{1} chickens at each level of GAT. The percentages

were different as a result of the culture conditions. Freshly collected lymphocytes from S1 line chickens have been assayed for level of CD4 and CD8 expression. Cells from B^{1H} chickens were the highest for CD4 expression and cells from B^{1L} chickens were the lowest for CD8 expression with no significant differences between the other S1 sublines for either marker (Munns 1990).

When results are examined within blood type, there are some interesting differences in the cultured cells. Within the B^{19} blood type there were differences in percentages of CD8-positive cells (suppressor type) between Ir-GAT^{H} and Ir-GAT^{L} chickens. The Ir-GAT^{L} had higher percentages of CD8-positive cells. There was no difference in CD8 levels between high and low GAT responders within the B^{1} blood type. Within the B^{1} blood type there were differences in percent CD4-positive cells between Ir-GAT^{H} and Ir-GAT^{L} chickens. The Ir-GAT^{L} cultures had higher levels of CD4 positive (helper type) cells at every level of GAT, but the difference in the means was only significant when the cells were cultured at the 10 μg GAT concentration.

It is possible that between the two blood types there are different mechanisms responsible for the differential response to GAT. The sublines within the S1 line arose from the same genetic group, but the groups with different Ir-GAT types were originated differently (some of the B^{1} chickens were drawn from populations that had been selected for antibody response to *S. pullorum*). In the B^{19} blood type, low response could be due to induction of suppressor T cells (CD8 positive). Within the B^{1} blood type high response could be due to the lack of suppression being

induced. Figure 1 (page 18) shows the relationships that exist between cells of the immune system. Small alterations in numbers of cells involved could cause effects that would be magnified by subsequent steps in the reaction.

Hála *et al.* (1991) reported differences in basal percent of CD4 and CD8 lymphocytes in congenic lines of chickens that differ in their resistance to Rous sarcoma virus-induced tumors and in their humoral immune response to various antigens. Hála *et al.* proposed that the differences in the ratio of CD4 to CD8 cells possibly contributed to RSV resistance, but offered no evidence or mechanisms. Cytotoxic T cells (that kill abnormal cells) also express the CD8 marker and the RSV-resistant line of chickens had higher levels of CD8 expression. In any event, small difference in immune cell populations can result in large observed differences in immunological traits. Gougeon and Theze (1983) attributed differences in immune response to GAT in mouse inbred lines to regulatory imbalances (or the ratio of T cell-mediated help to suppression) rather than discrete cellular defects (such as inability of APC to present antigen or T cells to respond). Under the appropriate conditions (by modifying T cell subset populations) non-responder T cells could proliferate in response to GAT and non-responder APC were able to present antigen.

Complementation Matings

Matings between two unrelated low GAT-responder sublines of chickens (GHs-B^{13} and S1-B^{19L}) resulted in progeny that responded to GAT challenge

by producing higher levels of antibody than either of the parental lines. The results from the F_1 generation gave the appearance that gene complementation was occurring. The progeny produced by backcrossing the F_1 to S1-B^{19L} and GHs-B^{13} chickens (and *inter se* F_1 matings) resulted in chickens of three blood types (B^{19}/B^{19}, B^{19}/B^{13}, and B^{13}/B^{13}). Secondary humoral GAT response was most significantly associated with the blood type of the progeny. The B^{13} homozygous chickens were low responders to GAT and the other two blood types were high responders to GAT. F_1 progeny produced by crossing S1-B^{1L} with S1-B^{19L} resulted in chickens that were low responders to GAT.

Non-MHC-linked genes were possibly responsible for the difference in the GAT response seen in the F_1 progeny. Dorf *et al.* (1974) examined the effects of non-MHC genes on GAT response in mice. They presented evidence that non-response or response was due to genes within or linked to the MHC and quantitative variation in response was due to non-MHC genes. That could explain the results seen with these chickens. The B^{13} chickens are non-responders to GAT, but they may carry complementary genes that allow the S1-B^{19L} chickens (that may be low responders and not non-responders) to produce higher levels of antibody. The within-S1 line crosses would not have introduced the complementary alleles that would result in high GAT response if those alleles were not in the S1 line or were present at a low frequency. This would not explain why progeny from F_1 chickens backcrossed to the S1-B^{19L} chickens had the same GAT response as chickens backcrossed to the GHs-B^{13} chickens, unless the F_2 progeny carried enough of the complementary alleles to result in high response to GAT. Since

high antibody response to antigen is invariably a dominant trait, the alleles probably would not have to be present in homozygous form to exert their effect. Dunnington *et al.* (1989) found that the antibody titer to sheep red blood cells (RBC) was determined by the MHC haplotype and the background genome. Although the sheep RBC is a more complex antigen than GAT, the results would seem to be dependent on the same mechanisms.

There are many examples of immune response differences that are not attributable to the MHC and show the importance of non-MHC genes. Palladino *et al.* (1977) found differences in resistance to Marek's disease among two chicken lines that were identical at the MHC (as shown by acceptance of transplanted skin grafts and other immunological tests). The same two lines also had quantitative differences in measurements of humoral and cellular immunity. Resistance to RSV-induced tumors in some inbred chicken lines has been shown to be under partial control of non-MHC genes that work to complement the MHC genes (Cutting *et al.* 1981). Gilmour *et al.* (1983) even identified the non-MHC genes that had allelic variants that resulted in different levels of susceptibility to RSV-induced tumors. The genes Gilmour found coded for alloantigens on T lymphocytes (*Ly-4* and *Th-1*).

Summary

The mechanisms that could result in high or low antibody response in the S1 line have been examined. T lymphocytes from Ir-GAT high or low chickens did not demonstrate any significant propensity to react to the antigen *in vitro*. The index scores (percentage of control) for the

sublines show a tendency for the cells from the Ir-GAT^{H} chickens to proliferate more than the cells from Ir-GAT^{L} chickens, but the differences were not statistically significant. The variation in response among chickens was very high regardless of MHC haplotype.

All of the experiments conducted *in vitro* could have benefitted from the availability of a reliable source of pure chicken IL-2. The IL-2 would have enable longer culture times and allowed more time for the differences between haplotypes to become evident. However, the IL-2 could have created more problems than it solved. If GAT were more strongly antigenic the proliferation *in vitro* may have been more pronounced, but the differences seen *in vivo* would probably also be different. Genetic control of differences in antibody response are more clearly seen when antigens of limited antigenic diversity are used. If the antigen stimulates more sub-populations of T lymphocytes, the resulting antibody production is likely to be larger and obscure differences due to immune response genes.

The antigen-presenting cells do not seem to be a cause of the humoral immune response differences in the SI chickens. Results indicate that the APC from Ir-GAT^{L} chickens present antigen better than the APC from Ir-GAT^{H} chickens to responder cells of either Ir-GAT type. If the antigen processing ability of the APC resulted in the antibody response differences this would not be the case. It seems unlikely that the antibody differences are due to antigen processing limitations. There is the possibility that the culture conditions used simply allowed the APC from Ir-GAT^{L} to function better in processing GAT *in vitro*.

Results from flow cytometry of CD4- and CD8-labelled cells indicate that immune response dichotomy is mediated by populations of effector T lymphocytes. The small proliferative response differences *in vitro* could obscure how different the CD4 and CD8 percentages between the haplotypes may be and the how much it determines the level of antibody response. Contrasting the two blood groups, there were differences between Ir-GAT^{H} and Ir-GAT^{L} chickens. The B^{1} chickens that were low responders to GAT had higher levels of CD4 positive (helper type) cells than the Ir-GAT^{L} chickens, at all GAT levels with differences statistically significant when the cells were cultured at the 10 μg level of GAT. The B^{19} chickens differed in percentage of CD8 positive cells according to Ir-GAT type at every level of GAT. The cells from B^{19L} chickens have a higher percentage of cells that carry the phenotypic marker of the suppressor T cell (CD8).

The results from the complementation matings point out the effects of the MHC or MHC-linked genes, as well as non-MHC-linked genes in controlling humoral immune response. There is evidence that response to GAT in chickens is similar to response to GAT in mice in that response or non-response is determined by genes within or linked to the MHC, but the amount of antibody produced may be determined by genes that are outside of the MHC.

Future Considerations

Although by serological and DNA study, Ir-GAT is not a classical MHC class I or class II gene, none of the results indicate that Ir-GAT is not linked to the MHC. There are still too many gaps in the chicken MHC

genomic map (where sequences and gene functions are not known) to allow for a complete understanding of immune response and how Ir-GAT is mediated. Many research groups are filling in the gaps in the chicken MHC, but it will take time.

Future work in understanding chicken immune response will benefit from advances in the basic ability to maintain cells *in vitro*. For example, there is progress being made toward developing recombinant chicken IL-2 which would enable the investigation of long term cultures of antigen-specific T cell clones.

Currently, the S1 sublines are being developed into congenic lines that differ for the MHC-bearing microchromosome. The congenic lines will provide additional information regarding the effects of complementary genes on immune response to GAT. It will be possible to determine MHC effects on antibody production in chickens that have non-MHC complementary genes in common.

The ability to identify genetic differences in populations through the techniques of DNA fingerprinting with multi-locus probes are currently being applied to investigate the differences in GAT response in the S1 line. This technique allows diagnostic segments of DNA on gels to be identified that are most likely linked to genes that contribute to a particular trait. The identified DNA segments can be isolated and further characterized by sequencing and comparison to existing gene data bases.

As the tools improve, so will the understanding of the mechanisms involved. The cellular mechanisms involved in immune response still cause dispute among immunologists working with the better studied mammalian

system. The results presented in this dissertation only begin to address the subject of immune response in chickens. No doubt the work of understanding immune response will continue long after this dissertation has turned to dust. The possibilities have not been exhausted and there are still questions that remain to be answered regarding immune response in chickens and how it influences the health of the chicken.

LITERATURE CITED

Adorini, L., and G. Doria. 1981. Defective antigen presentation by macrophages from mice genetically selected for low antibody response. Eur. J. Immunol. 11:984-989.

Ashwell, J. D. 1988. Are B lymphocytes the principal antigen-presenting cells in vivo?. J. Immunol. 140:3697-3700.

Benacerraf, B., and M. E. Dorf. 1977. Genetic control of immune responses and immune suppression by I-region genes. C. S. H. Symp. Quant. Biol. 41:465-475.

Benedict, A. A., L. W. Pollard, P. R. Abplanalp, P. H. Maurer, and W. E. Briles. 1975. Genetic control of immune response in chickens. I. Response to a terpolymer of poly($Glu^{60}Ala^{30}Tyr^{10}$) associated with the major histocompatibility complex. Immunogenetics 2:313-324.

Bierer, B. E., B. P. Sleckman, S. E. Ratnofsky, and S. J. Burakoff. 1989. The biologic roles of CD2, CD4, and CD8 in T-cell activation. Ann. Rev. Immunol. 7:579-599.

Biozzi, G., D. Mouton, C. Stiffel, and Y. Bouthillier. 1984. A major role of the macrophage in quantitative genetic regulation of immuno-responsiveness and antiinfectious immunity. Adv. Immunol. 36:189-233.

Bloom, S. and L. Bacon. 1985. Linkage of the major histocompatibility (*B*) complex and the nucleolar organizer in the chicken. J. Hered. 76:146-154.

Bluestein, H. G., I. Green, and B. Benacerraf. 1971. Specific immune response genes in guinea pigs. I. Dominant genetic control of immune responsiveness to copolymers of L-glutamic acid and L-alanine and L-glutamic acid and L-tyrosine. J. Exp. Med. 134:458-470.

Bourlet, Y., G. Béhar, F. Guillemot, N. Fiechem, A. Billault, A. M. Chaussé, and C. Auffray. 1988. Isolation of chicken major histocompatibility class II (*B-L*) β chain sequences: comparison with mammalian β chains and expression in lymphoid organs. EMBO J. 7:1031-1039.

Briles. W. E., W. H. McGibbon, and M. R. Irwin. 1950. On multiple alleles affecting cellular antigens in the chicken. Genetics 35:633-652.

Brown, M. A., L. A. Glimcher, E. A. Nielsen, W. E. Paul, and R. N, Germain. 1986. T-cell recognition of Ia molecules selectively altered by a single amino acid substitution. Science 231:255-258.

Bumstead, N., and M. J. Curtis. 1986. Identification of chicken major histocompatibility complex (B) haplotypes by an immunoblot technique. J. Immunol. Meth. 95:267-271.

Chan, M. M., C. H. Chen, L. L. Ager, and M. D. Cooper. 1988. Identification of the avian homologues of mammalian CD4 and CD8 antigens. J Immunol. 140:2133-2138.

Charron, D. J., M.-F. Aellen-schulz, J. St. Geme, H. A. Erlich, and H. O. McDevitt. 1983. Biochemical characterization of an invariant polypeptide associated with Ia antigens in human and mouse. Mol. Immunol. 20:21-32.

Chen, Y., and S. J. Lamont. 1991. Class I (B-F) major histocompatibility complex (MHC) restriction fragment length polymorphism (RFLP) analysis of thirteen highly inbred chicken lines and lines selected for MHC and antibody response. Poultry Sci. 70(suppl. 1):25. (Abstr.).

Cheng, S., Y. H. Hou, S. J. Lamont, and A. W. Nordskog. 1885. A pedigree analysis of the Iowa State White Leghorn S1 population bred primarily for immunogenetic studies. Poultry Sci. 63(suppl. 1):79. (Abstr.).

Cheng, S. and S. J. Lamont. 1988. Genetic analysis of immunocompetence measures in a White Leghorn chicken line. Poultry Sci. 67:989-995.

Cheng, S., M. F. Rothschild, and S. J. Lamont. 1991. Estimates of quantitative genetic parameters of immunological traits in the chicken. Poultry Sci. 70:(In Press).

Chi, D. S., and G. J. Thorbecke. 1987. Suppressor and other T-lymphocytes. pp. 161-177. *In*: Avian Immunology: Basis and Practice Vol.I. Eds.: A. Toivanen and P. Toivanen. CRC Press, Boca Raton. pp. 161-177.

Chu, Y. and R. R. Dietert. 1989. Monocyte function in chickens with hereditary muscular dystrophy. Poultry Sci. 68:226-232.

Clark, W. R. 1983. Genetic regulation of immune responsiveness. pp. 377-433. *In*: The experimental foundations of modern immunology. John Wiley & Sons, New York, NY.

Collins, W. M., W. E. Briles, R. M. Zsigray, W. R. Dunlop, A. C. Corbett, K. K. Clark, J. L. Marks, and T. P. McGrail. 1977. The *B*-locus (MHC) in the chickens: Association with the fate of RSV-induced tumors. Immunogenetics 5:333-343.

Cotter, P. F., R. L. Taylor, T. L. Wing, and W. E. Briles. 1987. Major histocompatibility (B) complex-associated differences in the delayed wattle reaction to *Staphylococcus* antigens. Poultry Sci. 66:203-205.

Crone, M., J. Jensenius, and C. Koch. 1981. Evidence for two populations of B-L (Ia-like) molecules encoded by the chicken MHC. Immunogenetics 13:381-391.

Cutting, J. A., D. H. Watanabe, F. R. Strebel, and R. A. McBride. 1981.

Complementing MHC- and non-MHC-linked genes and resistance to avian sarcoma virus-induced tumours in inbred lines of chickens. J. Immunogenetics 8:215-223.

DeFranco, A. L. 1988. Cell-cell interactions in the antibody response. Nature 334:199-200.

Dorf, M. E., E. K. Dunham, J. P. Johnson, and B. Benacerraff. 1974. Genetic control of the immune response: the effect of non-*H-2* linked genes on antibody production. J. Immunol. 112:1329-1336.

Dunnington, E. A., A. Martin, R. W. Briles, W. E. Briles, W. B. Gross, and P. B. Siegel. 1989. Antibody responses to sheep erythrocytes for White Leghorn chickens differing in haplotypes of the major histocompatibility complex (*B*). Animal Genetics 20:213-216.

Ewert, D. L., M. S. Munchus, C. H. Chen, and M. D. Cooper. 1984. Analysis of structural properties and cellular distribution of avian *Ia* antigen by using monoclonal antibody to monomorphic determinants. J. Immunol. 132:2524-2530.

Farrar, J. J., W. R. Benjamin, M. L. Hilfiker, M. Howard, W. L. Farrar, and J. Fuller-Farrar. 1982. The biochemistry, biology, and role of interleukin 2 in the induction of cytotoxic T cell and antibody-forming B cell responses. Immunol. Rev. 63:129-166.

Gavora, J. S. and J. L. Spencer. 1983. Breeding for immune responsiveness and disease resistance. An. Bld. Grps. Biochem. Gen. 14:159-180.

Gebriel, G. M. and A. W. Nordskog. 1983. Genetic linkage of subgroup C Rous sarcoma virus-induced tumor expression in chickens to the *Ir-GAT* locus of the *B* complex. J. Immunogenetics 10:231-235.

Gebriel, G., I. Y. Pevzner, and A. W. Nordskog. 1979. Genetic linkage between immune response to GAT and the fate of RSV-induced tumors in chickens. Immunogenetics 9:327-334.

Gershon, R. K., P. H. Maurer, and C. F. Merryman. 1973. A cellular basis for genetically controlled immunological unresponsiveness in mice: tolerance induction in T-cells. Proc. Natl. Acad. Sci. USA 70:250-254.

Gilmour, D. G., W. M. Collins, T. L. Fredericksen, B. Auclair, K. K. Clark, and W. E Urban, Jr.. 1983. Influence of non-MHC T lymphocyte alloantigens on regression of Rous sarcomas in the chicken. Immunogenetics 17:43-54.

Gougeon, M.-L., and J. Theze. 1983. MHC-linked Ir gene control of T cell response: GAT-specific proliferating T cells and helper T cells are elicited in non-responder mice immunized with soluble GAT. J. Immunol. 130:1521-1526.

Green, D. R., P. M. Flood, and R. K. Gershon. 1983. Immunoregulatory T-cell pathways. Ann. Rev. Immunol. 1:439-463.

Guillemot, F., A. Billault, and C. Auffrey. 1989a. Physical linkage of a guanine nucleotide-binding protein-related gene to the chicken major histocompatibility complex. Proc. Natl. Acad. Sci. USA 86:4594-4598.

Guillemot, F., A. Billault, O. Pourquie, G. Béhar, A.-M. Chaussé, R. Zoorob, G. Kreibich, and C. Auffrey. 1988. A molecular map of the chicken major histocompatibility complex: the class II β genes are closely linked to the class I genes and the nucleolar organizer. EMBO J. 7:2775-2785.

Guillemot, F., J. F. Kaufman, K. Skøedt, and C. Auffray. 1989. The major histocompatibility complex in the chicken. Trends in Genetics 5:300-304.

Guillemot, F., P. Turmel, D. Charron, N. LeDouarin, and C. Auffray. 1986. Structure, biosynthesis, and polymorphism of chicken MHC class II (*B-L*) antigens and associated molecules. J. Immunol. 137:1251-1257.

Hála, K., R. Boyd, and G. Wick. 1981. Chicken major histocompatibility complex and disease. Scand. J. Immunol. 14:607-616.

Hála, K., A. M. Chausse, Y. Bourlet, O. Lassila, V. Hasler, and C. Auffray. 1988. Attempt to detect recombination between *B-F* and *B-L* genes within the chicken *B* complex by serological typing, *in vitro* MLR, and RFLP analyses. Immunogenetics 28:433-438.

Hála, K., O. Vainio, J. Plachý, and G. Böck. 1991. Chicken major histocompatibility complex congenic lines differ in the percentages of lymphocytes bearing CD4 and CD8 antigens. Animal Genetics 22:279-284.

Hames, B. D.. 1981. An introduction to polyacrylamide gel electrophoresis. *In:* Gel Electrophoresis of Proteins- A Practical Approach. IRL Press, Oxford.

Hanson, M. P., J. N. van Zandt, and G. R. L. Law. 1967. Differences in susceptibility to Marek's disease in chickens carrying two different *B* locus blood group alleles. Poultry Sci. 46:1268. (Abstr.).

Harnett, M. M., and G. G. B. Klaus. 1988. G protein regulation of receptor signalling. Immunology Today 9:315-320.

Herber-Katz, E., D. Hansburg, and R. H. Schwartz. 1983. The *Ia* molecule of the antigen-presenting cell plays a critical role in immune response gene regulation of T cell activation. J. Mol. Cell. Immunol. 1:3-14.

Hunter, W. M.. 1973. Radioimmunoassay. pp.17.1-17.36. *In:* Handbook of Experimental Immunology. Ed.: D. M. Weir. Blackwell, Oxford.

Jaffe, W. P., and E. M. McDermid. 1962. Blood groups and splenomegaly in

chick embryos. Science 137:984.

Janeway, C. A.. 1983. Immune response genes, the problem of the non-responder, The *Ia* molecules of the antigen -presenting cell plays a critical role in immune response gene regulation of T cell activation. J. Mol. Cell. Immunol. 1:15-18.

Kaufman, J. F., C. Auffray, A. J. Korman, D. A. Shackelford, and J. Strominger. 1984. The class II molecules of the human and murine major histocompatibility complex. Cell 36:1-13.

Kimoto, M., and C. G. Fathman. 1980. Antigen-reactive T cell clones. I Transcomplementing hybrid I-A-region gene products function effectively in antigen presentation. J. Exp. Med. 152:759-770.

Kishimoto, T., and T. Hirano. 1988. Molecular regulation of B lymphocyte response. Ann. Rev. Immunol. 6:485-512.

Klein, J. 1986. Antigen-major histocompatibility complex-T cell receptors: Inquiries into the immunological ménage à trois. Immunol. Res. 5:173-190.

Klein, J., and J. M. Park. 1973. Graft-versus-host reactions across different regions of the *H-2* complex of the mouse. J. Exp. Med. 137:1213-1255.

Knudtson, K. L. 1987. Genetic associations of interleukin-2 levels in the chicken. M.S. Thesis. Iowa State University. 90 pp.

Knudtson, K. L., M. G. Kaiser, and S. J. Lamont. 1990. Genetic control of interleukin-2-like activity is distinct from that of mitogen response in chickens. Poultry Sci. 69:65-71.

Kroemer, G., F. Guillemot, and C. Auffray. 1990. Genetic organization of the chicken MHC. Immunol. Res. 9:8-19.

Kurt-Jones, E. A., D. Liano, K. A. HayGlass, B. Benacerraf, M.-S. Sy, and A. K. Abbas. 1988. The role of antigen presenting B cells in T cell priming in vivo: Studies of B cell deficient mice. J. Immunol. 140:3773-3788.

Laemmli, U. K.. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lambris, J. and M. Papamichail. 1980. A method for analyzing lymphocyte surface antigens. J. Immunol. Meth. 34:287-293.

Lamont, S. J., C. Bolin, and N. Cheville. 1987. Genetic resistance to fowl cholera is linked to the major histocompatibility complex. Immunogenetics 25:284-289.

Lassila, O., O. Vainio, and P. Matzinger. 1988. Can B cells turn on virgin T cells?. Nature 334:253-255.

Lee, R. W. H., and A. W. Nordskog. 1981. Role of the immune-response region of the *B* complex in the control of the graft-vs-host reaction in chickens. Immunogenetics 13:85-92.

Levine, B. B., A. Ojeda, and B. Benacerraf. 1963. Studies on artificial antigens. III. The genetic control of the immune response to hapten-poly-L-lysine conjugates in guinea pigs. J. Exp. Med. 118:953-957.

Lillehoj, H. S.. 1991. Lymphocytes involved in cell-mediated immune responses and methods to assess cell-mediated immunity. Poultry Sci. 70:1154-1164.

Lillehoj, H. S., E. P. Lillehoj, D. Weinstock, and K. A. Schat. 1988. Functional and biochemical characteristics of avian T lymphocyte antigens identified by monoclonal antibodies. Eur. J. Immunol. 18:2059-2065.

Littman, D. R.. 1987. The structure of the CD4 and CD8 genes. Ann. Rev. Immunol. 5:561-584.

Maccubbin, D. L., and L. W. Schierman. 1986. MHC-restricted cytotoxic response of chicken T cells: expression, augmentation, and clonal characterization. J. Immunol. 136:12-16.

MacDonald, H. R., and M. Nabholz. 1986. T-cell activation. Ann. Rev. Cell Biol. 2:231-253.

Marrack, P., and J. Kappler. 1986. The T cell and its receptor. Sci. American 254:36-45.

McDevitt, H. O., and A. Chinitz. 1969. Genetic control of the antibody response: Relationship between immune response and histocompatibility (*H-2*) type. Science 163:1207-1208.

McDevitt, H. O., D. B. Deak. D. C. Shreffler, J. Klein, J. H. Stimpfling, and G. D. Snell. 1972. Genetic control of the immune response. Mapping of the *Ir-1* locus. J. Exp. Med. 135:1259-1278.

Miggiano, V. C., I. Birgen, and J. R. L. Pink. 1974. The mixed lymphocyte reaction in chickens. Evidence for control by the major histo-compatibility complex. Eur. J. Immunol. 4:397-401.

Miggiano, V., M. North, A. Buder, and J. R. L. Pink. 1976. Genetic control of the response of chicken leukocytes to a T-cell mitogen. Nature 263:61-63.

Morgan, T. J., H. S. Lillehoj, B. G. Sanders, and K. Kline. 1990. Character-ization and developmental expression of the chicken *B-G* heterodimer.

Dev. Comp. Immunol. 14:425-437.

Morrow, P. R., and H. Abplanalp. 1981. Genetic control of T-lymphocyte mitogenesis in chickens. Immunogenetics 13:189-200.

Mosmann, T.. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferative and cytotoxicity assays. J. Immunol. Meth. 65:55-63.

Mouton, D., Y. Bouthillier, J. C. Mevel, and G. Biozzi. 1984. Genetic selection for antibody responsiveness in mice: Further evidence of inverse modification of macrophage catabolic activity without alteration of the expression of T-cell-mediated immunity. Ann. Immunol. (Inst. Pasteur) 135D:173-185.

Munns, P. L. 1990. Lymphocyte subpopulations in chickens with regressing or progressing tumors induced by Rous sarcoma virus. M.S. Thesis. Iowa State University. 71 pp.

Nabholz, M., and H. R. MacDonald. 1983. Cytolytic T lymphocytes. Ann. Rev. Immunol. 1:273-306.

Neer, E. J., and D. E. Clapham. 1988. Roles of G protein subunits in transmembrane signalling. Nature 333:129-134.

Nordskog, A. W., I. Y. Pevzner, C. L. Trowbridge, and A. A. Benedict. 1977. Immune response and adult mortality associated with the *B* locus in chickens. Adv. Exp. Med. Biol. 88:245-256.

Nordskog, A. W., W. A. Rishell, and D. M. Briggs. 1973. Influence of *B* locus blood groups on adult mortality and egg production in the White Leghorn chicken. Genetics 75:181-189.

Palladino, M. A., D. G. Gilmour, A. R. Scafuri, H. A. Stone, and G. J. Thorbecke. 1977. Immune response differences between two inbred chicken lines identical at the major histocompatibility complex. Immunogenetics 5:253-259.

Paul, W. E., and J. Ohara. 1987. B-cell stimulatory factor-1/Interleukin-4. Ann. Rev. Immunol. 5:429-459.

Pazderka, F., B. M. Longenecker, G. R. J. Law, and R. F. Ruth. 1975a. The major histocompatibility complex of the chicken. Immunogenetics 2:101-130.

Pazderka, F., B. M. Longenecker, G. R. J. Law, H. A. Stone, and R. F. Ruth. 1975. Histocompatibility of chicken populations selected for resistance to Marek's disease. Immunogenetics 2:93-100.

Pevzner, I. Y., I. Kujdych, and A. W. Nordskog. 1981. Immune response and

disease resistance: II. Marek's disease and immune response to GAT. Poultry Sci. 60:927-932.

Pevzner, I. Y., E. O. Mills, and F. K. Mack. 1989a. Genetic control of immune response to GAT in meat and egg-type commercial chickens. Poultry Sci. 68(suppl. 1):113. (Abstr.).

Pevzner, I. Y., E. O. Mills, and F. K. Mack. 1989b. Disease resistance and immune response to GAT in meat and egg-type chickens. Poultry Sci. 68(suppl. 1):113. (Abstr.).

Pevzner, I., A. W. Nordskog, and M. L. Kaeberle. 1975. Immune response and the *B* blood group locus in chickens. Genetics 80:753-759.

Pevzner, I. Y., C. L. Trowbridge, and A. W. Nordskog. 1977. Selection for high and low antibody response to *Salmonella pullorum* in chickens. Genetics 86:48-49.

Pevzner, I. Y., C. L. Trowbridge, and A. W. Nordskog. 1978. Recombination between genes coding for immune response and the serologically determined antigens in the chicken B system. Immunogenetics 7:25-33.

Pevzner, I. Y., C. L. Trowbridge, and A. W. Nordskog. 1979. *B*-complex genetic control of immune response to HSA, (T,G)-A-L, GT and other substances in chickens. J. Immunogenetics 6:453-460.

Pierce, C. W., C. M. Sorenson, and J. A. Kapp. 1988. Identification of suppressor T cells in virgin non-responder spleen cells responsible for primary unresponsiveness to L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT). J. Immunol. 141:64-70.

Pink, J. R. L., W. Droege, K. Hála, V. C. Maggiano, and A. Ziegler. 1977. A three-locus model for the chicken major histocompatibility complex. Immunogenetics 5:203-216.

Pink, J. R. L., M. W. Kieran, A. M. Rijnbeek, and B. M. Longenecker. 1985. A monoclonal antibody against chicken MHC class I (*B-F*) antigen. Immunogenetics 21:293-297.

Pink, J. R., and V. C. Miggiano. 1977. Complementation between genetic variants affecting the response of chicken leukocytes to concanavalin A. J. Immunol. 119:1796-1799.

Pitcovski, J., S. J. Lamont, A. W. Nordskog, and C. W. Warner. 1989. Analysis of *B-G* and immune response genes in the Iowa State University S1 chicken line by hybridization of sperm deoxyribonucleic acid with a major histocompatibility complex class II probe. Poultry Sci. 68:94-99.

Rees, M. J., and A. W. Nordskog. 1981. Genetic control of serum immunoglobulin G levels in the chicken. J. Immunogenetics 8:425-431.

Salomonsen, J., K. Skjødt, M. Crone, and M. Simonsen. 1987. The chicken erythrocyte-specific MHC antigen. Characterization and purification of the *B-G* antigen by monoclonal antibodies. Immunogenetics 25:373-382.

SAS Institute Inc.. (1985). SAS/STAT™ Guide for Personal Computers, Version 6 Edition. SAS Institute Inc., Cary, NC.

Schauenstein, K., A. Globerson, and G. Wick. 1982. Avian lymphokines: 1. Thymic cell growth factor in supernatants of mitogen stimulated spleen cells. Dev. Comp. Immunol. 6:533-540.

Schierman, L. W. and A. W. Nordskog. 1961. Relationship of blood type to histocompatibility in chickens. Science 1334:1008-1009.

Schierman, L. W., D. H. Watanabe, and R. A. McBride. 1977. Genetic control of Rous sarcoma regression in chickens: Linkage with the MHC. Immunogenetics 5:325-332.

Schwartz, R. H., L. A. Matis, and Z. Kovac. 1985. Histocompatibility molecules as immune response gene products. *In:* Cell Biology of the Major Histocompatibility Complex. pp. 273-282. Eds.: B. Pernis and H. J. Vogel. Academic Press, New York, NY.

Shreffler, D. C., and C. S. David. 1975. The *H-2* major histocompatibility complex and the *I* immune response region: genetic variation, function, and organization. Adv. Immunol. 20:175-195.

Skjødt, K., C. Koch, M. Crone, and M. Simonsen. 1985. Analysis of chickens for recombination within the MHC (*B*-complex). Tissue Antigens 25:278-282.

Steadham, E. M., S. J. Lamont, I. Kujdych, and A. W. Nordskog. 1987. Association of Marek's disease with *Ea-B* and immune response genes in subline and F_2 populations of the Iowa State S1 Leghorn line. Poultry Sci. 66:571-575.

Thomas, D. W., and M. D. Hoffman. 1982. Lack of a macrophage defect in presentation of antigens under Ir gene control. J. Immunol. 128:780-783.

Unanue, E. R.. 1984. Antigen-presenting function of the macrophage. Ann. Rev. Immunol. 2:395-428.

Vainio, O., C. Koch, and A. Toivanen. 1983. Genetic control of T-B cell interaction in the chicken. Transplant. Proc. 15:174-176.

Vainio, O., and O. Lassila. 1989. Chicken T cells: Differentiation antigens and cell-cell interactions. Poultry Biol. 2:97-102.

Vainio, O., T. Veromaa, E. Eerola, P. Toivanen, M. J. H. Ratcliffe. 1988. Antigen-presenting cell-T cell interaction in the chicken is MHC class II antigen restricted. J. Immunol. 140:2864-2868.

Vitetta, E. S., S. Baur, and J. W. Uhr. 1971. Cell surface immunoglobulin. II. Isolation and characterization of immunoglobulin from mouse splenic lymphocytes. J. Exp. Med. 134:424-264.

von Boehmer, H., W. Haas, and N. Jerne. 1978. Major histocompatibility complex-linked immune responsiveness is acquired by lymphocytes of low responder mice differentiating in thymus of high-responder mice. Proc. Nat. Acad. Sci. (USA) 75:2439-2442.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the members of my committee for wading through this dissertation. After writing a dissertation, I have a profound appreciation for what it must take to read one. Dr. Susan Lamont deserves special thanks for her support and guidance while the research was being done and while the results were written.

It has been my good fortune to work with people who were not only knowledgeable, but fun to work with and for. The many people who have passed through the poultry laboratory during my time here all receive my thanks. Mike Kaiser, Ron Kean, Martha Jeffrey, and Ann Shuey all deserve mention for tolerating me for the last six months.

Most greatly I wish to thank my family. My parents, my mother-in-law, my sisters, and my brothers-in-law have all contributed in ways I cannot begin to thank them for. If anything has made this trip into higher education any easier it has been knowing that my family was always there.

Lastly, I thank my wife, Shari and daughter, Marni for their ability to make me smile and for reminding me that there are some things that are more important. No man could ever boast of a more supportive and loving family.

APPENDIX

Association of Marek's Disease with Ea-B and Immune Response Genes in Subline and F_2 Populations of the Iowa State S1 Leghorn Line[1]

E. M., Steadham, S. J. Lamont[2], I. Kujdych[3], and A. W. Nordskog

Department of Animal Science
Iowa State University
Ames, Iowa 50011

Running Title: Genetics of Marek's Disease Resistance

Section Preference: Breeding and Genetics

---

[1]Joint contribution from the Iowa Agricultural and Home Economics Experiment Station, Ames, IA, Project 2237 (Journal Paper J-12036), and Cook College, New Jersey Agricultural Experiment Station, Project 06220 (Journal Paper D-06220-02-85), in cooperation with the Northeastern States Regional Poultry Breeding Project, NE-60. Supported in part by NIH Grant AI 12746.
[2]To whom correspondence should be addressed.
[3]Cook College, Poultry Health Laboratory, Rutgers, The State University of New Jersey, Vineland, NJ 08360.

Abstract

Chickens from the Iowa State S1 White Leghorn line, selected for characteristics of Ea-B serotype, humoral immune response to glutamic acid-alanine-tyrosine (Ir-GAT), and response to Rous sarcoma virus (RSV)-induced tumors, were evaluated for genetic resistance to Marek's disease (MD). In the first two trials, sublines that were triple homozygous for the 3 traits were challenged with MD virus. Birds of the $\underline{B}^1\underline{B}^1$ blood type were significantly ($P<.001$) more resistant to MD than chickens of the $\underline{B}^{19}\underline{B}^{19}$ blood type. High responders to GAT were significantly ($P<.001$) more resistant to MD virus than low responders. The RSV classification had no detectable association with MD resistance. Chickens challenged with MD virus in the third trial were an F_2 population produced from *inter se* matings of S1 chickens heterozygous for the three traits under selection. Data from this trial confirmed the increased MD resistance of chickens possessing the $\underline{B}^1\underline{B}^1$ blood type when associated with genes encoding high immune response to GAT.

1987 Poultry Science 66:571-575.

(Key words: Marek's disease, major histocompatibility complex, immune response, genetic resistance, glutamic acid-alanine-tyrosine)

Introduction

The effectiveness of selection for Marek's disease (MD) resistance in poultry is well established. Pioneering work of Hutt and Cole (1948) first established the feasibility of such selection. Later work by Cole (1968) demonstrated significant differences in MD susceptibility after only two generations of selection, suggesting simple genetic control. Hanson et al. (1967) first reported an association between a B locus blood group allele and MD resistance. Cole's MD-resistant line N was found to be uniformly homozygous for the B^{21} allele (Pazderka et al., 1975; Briles et al., 1977). Other researchers have also identified B locus alleles associated with MD resistance or susceptibility (Pazderka et al., 1975; Longenecker et al., 1976; Bacon and Witter, 1980; Longenecker and Mosmann, 1981).

The B complex is the chicken major histocompatibility complex (MHC) for which the erythrocyte alloantigen B (Ea-B) serves as a useful genetic marker (Schierman and Nordskog, 1961). Pevzner et al., (1981) reported that MD resistance was associated with the immune response region identified by humoral immune response to glutamic acid-alanine-tyrosine (Ir-GAT) within the MHC of the Iowa State University S1 line. Briles et al. (1983) and Plachy et al. (1984) mapped a gene controlling MD resistance to the B-F region of the MHC. The B-F region, by Plachy's definition, contains the immune response region (B-L); this finding is similar to those of Pevzner et al. (1981). Briles and coworkers (1983), mapping MD resistance by using recombinants between B-F and B-G regions, did not comment on linkage of the B-L and B-F regions in their crossover.

Only one generation of S1 line chickens segregating for both Ea-B and Ir-GAT was challenged with MD virus in the study of Pevzner et al. (1981). Selection for the ability to regress RSV-induced tumors has also been conducted in the S1 sublines and was not addressed in Pevzner's work. The present study reports further analysis of MD susceptibility in the Iowa State S1 Leghorn line with special reference to the association with Ea-B, immune response to GAT, and RSV-tumor response.

Materials and Methods

Genetic Stocks. The S1 line originated from a cross of two commercial inbred lines (Nordskog et al., 1973). It has been maintained since 1978 in sublines selected for homozygous Ea-B serotype (B^1B^1 or $B^{19}B^{19}$) and for the humoral immune response to the amino acid polymer, GAT (Pevzner et al., 1978). More recently, the sublines were further selected for resistance or susceptibility to Rous sarcoma virus (RSV)-induced tumors from information on sibling tests. This study used 8 sublines, representing all triple homozygous combinations of Ea-B (B^1 or B^{19}), Ir-GAT (high or low) and RSV response (regressor or progressor). Each generation was produced from five males of each subline which were mated by artificial insemination to four or five nonsib hens of the same subline. The breeders were selected to represent the appropriate Ea-B and RSV type, and possess optimum response to GAT (either high or low, as appropriate). Ten to 20% of the progeny in each generation were selected as breeders. The inbreeding coefficients of Trial 2 sublines averaged .40 (Cheng et al., 1985).

The first two trials utilized 2 subsequent generations of the 8 sublines described previously. The third trial of S1 chickens utilized an F_2 population, derived from matings of B^{19} Ir-GAT high/B^1 Ir-GAT low X B^{19} Ir-GAT high/B^1 Ir-GAT low and B^{19} Ir-GAT low/B^1 Ir-GAT high X B^{19} Ir-GAT low/B^1 Ir-GAT high matings (Figure 1). No RSV designation was assigned to the chickens of this population, since 3 different genotypes could be generated from each mating. Two of the six haplotypes produced were phenotypically identical ($\underline{B}^1\underline{B}^{19}$ Ir-GAT high) since high response to GAT is a dominant trait. In some birds, however, the Ir-GAT high gene was linked with $\underline{B}^{19}$, and in others Ir-GAT was linked with $\underline{B}^1$. The data from the phenotypically identical groups were pooled because the incidences of MD among them were not significantly different. This yielded 5 genetic groups for analysis.

Marek's Disease Challenge. Challenge tests for MD were carried out at the Rutger's Poultry Health Laboratory under the supervision of Dr. I. Kujdych. Two-day old chicks were inoculated intraperitoneally with .25 ml (2,500 PFU) of the JM isolate of MD virus (provided by R. Witter, RPRL, East Lansing, MI). The chicks were housed together in a floor pen and received feed and water ad libitum. All birds were necropsied for evidence of gross MD lesions at death or at the termination of the studies at 17 weeks.

Chickens in this study were classified as susceptible to Marek's disease if they either died from MD before 17 weeks or survived to the termination of the experiment and showed MD lesions upon necropsy. Analysis of variance was performed using percentage susceptible to MD

within each group as the variable and traits of selection as sources of variation. Significance of differences between genetic groups were tested on the basis of the X^2 statistic.

Results and Discussions

The results from trials 1 and 2 are reported individually and are also shown pooled because the difference between these trials was not significant (Tables 1 and 2). The effects of Ea-B, Ir-GAT, and their interaction were highly significant sources of variation in trials 1 and 2 (Table 1). Classification for RSV response had no effect on MD susceptibility, and is not included in the data presented in Table 2. The $\underline{B}^1\underline{B}^1$ Ir-GAT high sublines were the most resistant to MD (Table 2 and Figure 2). Association of Ir-GAT with resistance to MD in the S1 line was earlier reported by Pevzner *et al.* (1981). The association of MD resistance with the B-F/B-L subregion of the MHC is also congruent with findings of Briles *et al.* (1983) and Plachy *et al.* (1984).

In this first investigation of association between RSV response and MD resistance in the S1 line, chickens differing in RSV classification (progressor or regressor) did not differ in incidence of MD (Table 1 and Figure 2). Direct selection for RSV response seemed not to influence MD resistance, even though both are virally-induced tumors. Calnek *et al.* (1975) also reported that the incidence of MD and susceptibility to progressive RSV-induced tumors were not necessarily correlated.

In trial 3, a challenge of F_2 birds, the $\underline{B}^1\underline{B}^1$ Ir-GAT high group was significantly different from all the other groups in incidence of MD

(Table 2 and Figure 3). Analysis of variance by haplotype showed that the genetic group (combination of Ea-B genotype and Ir-GAT phenotype) was a significant source of variation in incidence of MD (Table 3). Analysis of Ea-B X Ir-GAT interaction could not be carried out in trial 3, as it had been in trials 1 and 2, because of the heterozygous individuals tested in trial 3.

Data from all three trials show that $\underline{B}^1\underline{B}^1$ Ir-GAT high chickens are more resistant to MD than either $\underline{B}^{19}\underline{B}^{19}$ or Ir-GAT low chickens. These results are congruent with those of Pevzner et al. (1981). The relatively greater resistance, compared to other haplotypes, of the $\underline{B}^1\underline{B}^1$ Ir-GAT high haplotype to MD has persisted for several generations in the S1 population. Trial 3 of this experiment extends results of Pevzner et al. (1981) by testing an F_2 population and definitively linking resistance to MD with genes controlling immune response to GAT.

The difference in relative resistance to MD of the $\underline{B}^1\underline{B}^1$ Ir-GAT low birds between the sublines of trials 1 and 2 and the F_2 population of trial 3 suggests a role for complementary gene loci outside of the MHC. Fredericksen et al. (1977) reported 2 non-MHC loci that code for lymphocyte alloantigens influencing MD resistance. Possibly, the generation of an F_2 population from the homozygous sublines resulted in a reassortment of complementary loci. Future MD challenge of congenic lines of chickens with the S1 MHC haplotypes-lines that are currently in development- should help to clarify the role of MHC versus non-MHC loci in MD resistance.

References

Bacon, L. C., and R. L. Witter, 1980. B^2 determines resistance of Marek's disease tumor in contrast to B^5 in $(6_3 \times 15_1)F_5$ chickens. Fed. Proc. 39:938. (Abstr.)

Briles, W. E., R. W. Briles, R. E. Tafts, and H. A. Stone, 1983. Resistance to a malignant lymphoma in chickens is mapped to a subregion of major histocompatibility (B) complex. Science 219:977-979.

Briles, W. E., R. W. Briles, and R. K. Cole, 1977. Marek's disease: Effects of B histocompatibility alloalleles in resistant and susceptible chicken lines. Science 195:193-195.

Calnek, B. W., D. A. Higgins, and J. Fabricant, 1975. Rous sarcoma regression in chickens resistant or susceptible to Marek's disease. Avian Dis. 19:473-482.

Cheng, S., Y. H. Hou, S. J. Lamont, and A. W. Nordskog, 1985. A pedigree analysis of the Iowa State White Leghorn S1 population bred primarily for immunogenetics studies. Poultry Sci. 64 (Suppl. 1):79. (Abstr.)

Cole, R. K., 1968. Studies on genetic resistance to Marek's disease. Avian Dis. 12:9-28.

Fredericksen, T. L., B. M. Longenecker, F. Pazderka, D. G. Gilmour, and R. F. Ruth, 1977. A T-cell antigen system of chickens: Ly-4 and Marek's disease. Immunogenetics 5:535-552.

Hanson, M. P., J. N. van Zandt, and G. R. J. Law, 1967. Differences in susceptibility to Marek's disease in chickens carrying two different B locus blood group alleles. Poultry Sci. 46:1268. (Abstr.).

Hutt, F. B., and R. K. Cole, 1948. The development of strains genetically resistant to avian lymphomatosis. Pages 719-723 *in:* Proc. of Eighth World's Poultry Congr., Copenhagen, Denmark.

Longenecker, B. M., F. Pazderka, J. S. Gavora, J. L. Spencer, and R. F. Ruth, 1976. Lymphoma induced by herpes virus: resistance associated with a major histocompatibility gene. Immunogenetics 3:401-407.

Longenecker, B. M., and T. R. Mosmann, 1981. Structure and properties of the major histocompatibility complex of the chicken. Speculations on the advantages and evolution of polymorphism. Immunogenetics 13:1-13.

Nordskog, A. W., W. A. Rishell, and D. M. Briggs, 1973. Influence of B locus blood groups on adult mortality and egg production in the White Leghorn. Genetics 5:181-189.

Pazderka, F., B. M. Longenecker, G. R. J. Law, H. A. Stone, and R. F.

Ruth, 1975. Histocompatibility of chicken populations selected for resistance to Marek's disease. Immunogenetics 2:93-100.

Pevzner, I. Y., I Kujdych, and A. W. Nordskog, 1981. Immune response and disease resistance in chickens: 2. Marek's disease and immune response to GAT. Poultry Sci. 60:927-932.

Pevzner, I. Y., C. L. Trowbridge, and A. W. Nordskog, 1978. Recombination between genes coding for immune response and the serologically determined antigens in the chicken B system. Immunogenetics 7:25-33.

Plachy, J., V. Jurajda, and V. Benda, 1984. Resistance to Marek's disease is controlled by a gene within the B-F region of the chicken major histocompatibility complex in Rous sarcoma regressor or progressor inbred lines of chickens. Folia Biol. (Praha) 30:251-258.

Schierman, L. W., and A. W. Nordskog, 1961. Relationship of blood type to histocompatibility in chickens. Science 134:1008-1009.

Table 1. *Analysis of variance of factors affecting the incidence of Marek's disease: Trials 1 and 2*[1]

Source	Trial 1 df	Trial 1 MSx10^{-3}	Trial 2 df	Trial 2 MSx10^{-3}	Pooled data, Trials 1 and 2 df	Pooled data, Trials 1 and 2 MSx10^{-3}
Trial	...	...	...	...	1	5
Sex	1	3	1	67	1	49
Ea-B[2]	1	1450**	1	1233**	1	2679**
Ir-GAT[3]	1	330*	1	315**	1	645**
RSV[4]	1	62	1	5	1	52
Ea-B*Ir-GAT	1	143	1	115*	1	257*
Other 1st order interactions[5]	5	51	5	20	5	24
Error	5	18	5	6	20	20

[1]Mean square.

[2]Ea-B = Erythrocyte alloantigen B.

[3]Ir-GAT = Immune response to glutamic acid-alanine-tyrosine.

[4]RSV = Rous sarcoma virus.

[5]Tested individually, pooled for clarity.

*P<.01

**P<.001

Table 2. *Incidence of Marek's disease (MD+) in the S1 line*[1].

B Genotype	Ir-GAT[2] Phenotype[3]	Trial 1 # at risk	Trial 1 % MD+	Trial 2 # at risk	Trial 2 % MD+	Pooled data, Trials 1 & 2 # at risk	Pooled data, Trials 1 & 2 % MD+	Trial 3 # at risk	Trial 3 % MD+
$B^{19}B^{19}$	High	118	37.3[a]	139	45.3[a]	257	41.6[a]	56	42.1[a]
$B^{19}B^{19}$	Low	87	87.4[b]	81	80.2[b]	168	83.9[b]	77	43.0[a]
$B^{1}B^{1}$	High	69	4.3[c]	125	6.4[c]	194	5.7[c]	49	10.2[b]
$B^{1}B^{1}$	Low	143	11.2[c]	67	16.4[c]	210	12.9[d]	39	46.2[a]
$B^{1}B^{19}$	High	...	...	...	...	...	...	112[4]	40.2[a]

[a-d]Groups within a column with different superscripts are significantly different in incidence of MD ($P<.05$).

[1]Rous sarcoma virus classification had no effect and is ignored for presentation of genetic group means.

[2]Ir-GAT = Immune response to glutamic acid-alanine-tyrosine.

[3]Ir-GAT High dominant over Ir-GAT Low.

[4]Includes B^{1} Ir-GAT High/B^{19} Ir-GAT Low and B^{1} Ir-GAT Low/B^{19} Ir-GAT High genotypes.

Table 3. *Analysis of variance of factors affecting the incidence of Marek's disease: Trial 3*[1]

Source of variation	df	MS X 10^{-3}
Sex	1	.4
Ea-B[2], Ir-GAT[3]	4	46*
Error	4	3

[1]Mean square.

[2]Ea-B = Erythrocyte alloantigen B.

[3]Ir-GAT = immune response to glutamic acid-alanine-tyrosine. Ea-B and Ir-GAT classifications are combined as one factor for analysis (see Materials and Methods section for description of the five genetic groups).

*P<.01.

Figure Legends

Figure 1. Pedigree (without inclusion of RSV classification) of the population challenged with Marek's disease (MD) virus. H and L represent immune response to glutamic acid-alanine-tyrosine (Ir-GAT) (High and Low, respectively). Chickens representative of the sublines of the P_1 generation were challenged in the first two trials. The heterozygous chickens (F_1) were not challenged with MD virus. The numbers of chicks of the F_2 population challenged in the third trial are given. The F_2 haplotypes within the box were phenotypically identical (B^1B^{19} Ir-GAT High).

Figure 2. Incidence of Marek's disease (MD) in S1 line chickens challenged with JM strain of MD virus. Pooled results from trials 1 and 2. Eight sublines are represented, based on erythrocyte alloantigen B (Ea-B), immune response to glutamic acid-alanine-tyrosine (Ir-GAT), and Rous sarcoma virus (RSV) response (regressor or progressor).

Figure 3. Incidence of Marek's disease (MD) in S1 line F_2 chickens (erythrocyte alloantigen B (Ea-B) and immune response to glutamic acid-alanine-tyrosine (Ir-GAT)), Trial 3. Five groups are represented as described in Materials and Methods. The B^1B^{19} Ir-GAT High represents the pooled response of two haplotypes.

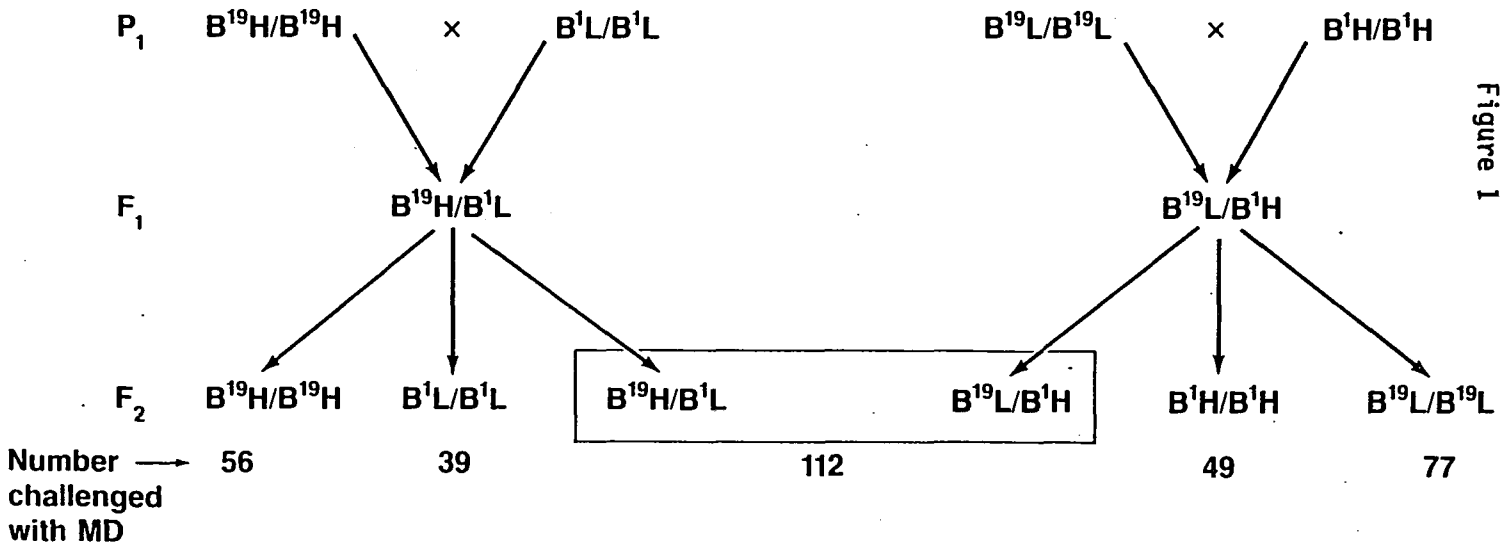

P1
B19H/B19H
×
B1L/B1L
B19L/B19L
×
B1H/B1H
F1
B19H/B1L
B19L/B1H
F2
B19H/B19H
B1L/B1L
B19H/B1L
B19L/B1H
B1H/B1H
B19L/B19L
Number challenged with MD
56
39
112
49
77

Figure 1

Figure 2

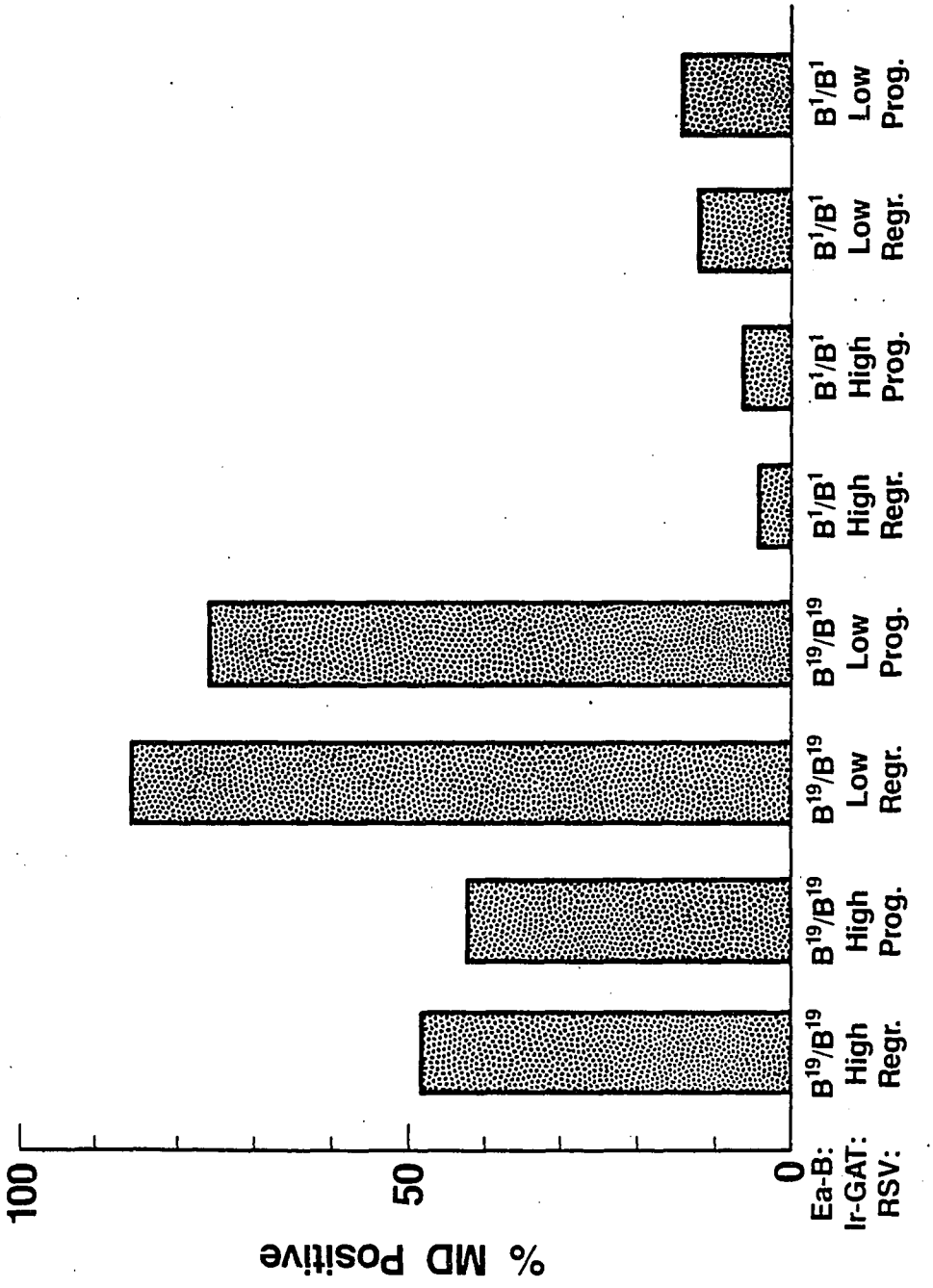
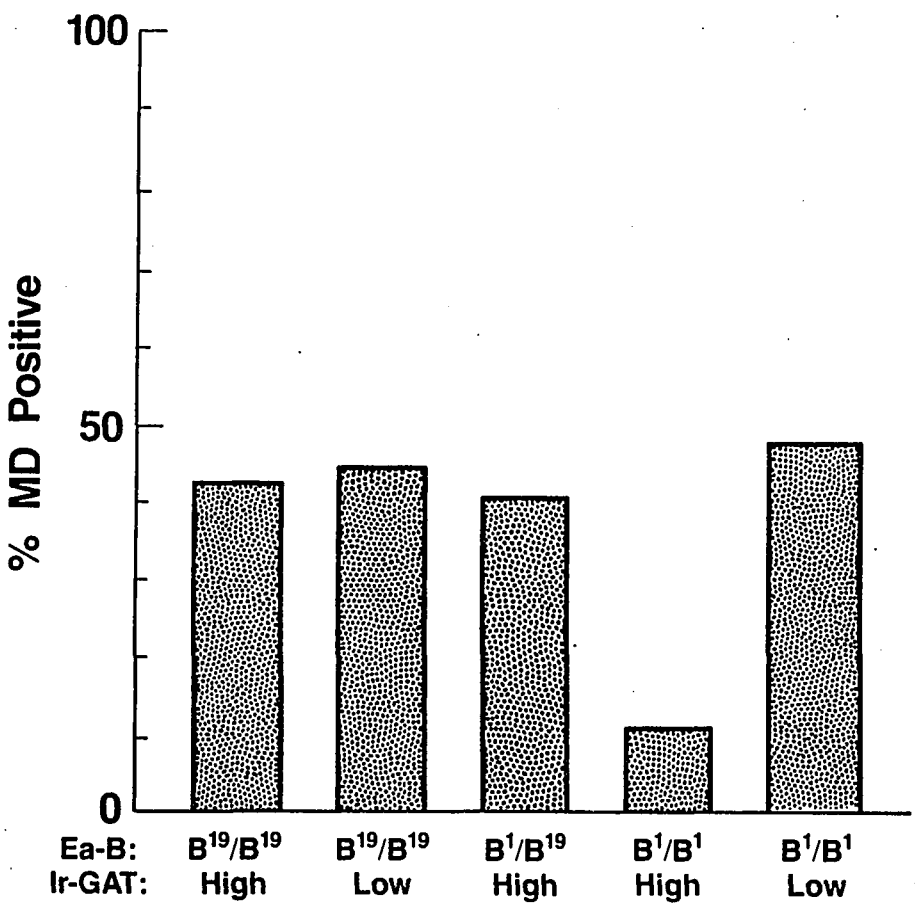

% MD Positive
100
50
0
Ea-B: B19/B19 B19/B19 B19/B19 B19/B19 B1/B1 B1/B1 B1/B1 B1/B1
Ir-GAT: High High Low Low High High Low Low
RSV: Regr. Prog. Regr. Prog. Regr. Prog. Regr. Prog.

Figure 3


100
50
0
% MD Positive
Ea-B:
Ir-GAT:
B^{19}/B^{19} High
B^{19}/B^{19} Low
B^{1}/B^{19} High
B^{1}/B^{1} High
B^{1}/B^{1} Low